附录 2：国内良知学者 13 项研究揭示草甘膦损伤蛋白质和脂质、致细胞凋亡和坏死、对肝细胞具有明显的损伤作用、致突变、致生殖毒性，具有对人类后代致出生缺陷强大能力！

Attachment 2: Thirteen studies by Chinese conscience scholars reveal that glyphosate damages protein and lipids, causes cell apoptosis and necrosis, shows obvious damage to liver cells, is mutagenic, causes reproductive toxicity, and has strong ability to cause birth defects!

要点概述

Summary of Main Points

1）邬惠琼（1996）：草甘膦与试验鼠"肝微粒体蛋白含量明显减少…蛋白含量减少可能与肝细胞受损致使合成蛋白能力下降有关；

1) Wu Hui-qiong (1999): **Glyphosate and test rat "Liver microsomal protein content decreased significantly … protein content reduction might be associated with protein synthesis ability reduction caused by impaired liver cell damage."**

2）耿德贵等（2000）：对黄鳝具有明显的遗传学损伤作用"

2) Geng De-gui et al. (2000): Causes obvious genetic damage to yellow eel"

3）南旭阳（2001）：不同浓度草甘膦药物"对蟾蜍的红细胞微核率和核异常率均有一定程度的影响

3) Nan Xuyang (2001) Different concentration of glyphosate "causes certain degree of effect on RBC micronucleus rate and the rate of nuclear anomalies of toads"

4）南旭阳（2002）：对鲫鱼的血红蛋白、红细胞和白细胞影响较大

4) Nan Xuyang (2002): "causes rather large effect on Crucian carp hemoglobin, red blood cells and white blood cells, with rather obvious time duration effect."

5）南旭阳等（2003）：对泥鳅具有一定的生理毒性"

5) Nan Xuyang et al. (2003): **"causes certain degree of biological toxicity to loaches".**

6）康菊芳等（2008）：对小鼠具有生殖毒性并具有一定的致突变作用

6) Kang Jufang et al. (2008): **“causes reproduction toxicity to mice with a certain mutagenic effect”.**

7）王非（2008）：能引起人肝细胞存活率下降,细胞膜通透性增加,抑制细胞离子转运,诱发 DNA 损伤,线粒体膜电位降低,Cyt C、AIF 等凋亡因子泄漏,使细胞产生凋亡和坏死,对肝细胞具有明显的损伤作用。

7) Wang Fei (2008): **“Can lead to liver cell survival rate decrease, cell membrane permeability increase, inhibit cell ion transport, induce DNA damage, mitochondrial membrane potential decreased, leakage of Cyt C, AIF apoptosis factors, causes cell apoptosis and necrosis, obvious damage to liver cells”.**

8）黄婷（2010）：可引起小鼠精子数目减少、精子畸形率增加,以及附睾和睾丸重量及其系数下降,提示农达对雄性小鼠具有明显的生殖毒性作用。

8) Huang Ting (2010): Could cause mice sperm number reduce, sperm deformity rate increase, epididymis and testis weight and coefficient decline, suggesting Roundup causes obvious reproductive toxicity in male mice”.

9）李娇等（2010）：草甘膦对海胆胚胎各发育期具有一定的急性毒性

9) Li Qiao et al. (2010): “Glyphosate causes certain acute toxicity to sea urchin embryos during different phases of development”.

10）赵伟等（2011）：草甘膦能降低小鼠的总抗氧化能力,损伤蛋白质和脂质,造成机体的氧化损伤,导致各种疾病的发生。

10) Zhao Wei et al. (2011): **“Glyphosate can reduce total antioxidant capacity, damage protein and lipid, cause oxidative damage of the body, cause development of various diseases”.**

11）俞慧等（2012）：草甘膦对小鼠具有生殖毒性并具有一定的致突变作用

11) Yu Hui et al. (2012): **“Glyphosate causes reproductive toxicity to mice and has certain mutagenic effect”.**

12）曾明等（2014）：研究表明,60-180 mg•L-1 浓度草甘膦对 GC-1 细胞有明显的损伤作用,其机制可能是草甘膦诱导氧化应激,导致细胞通透性增加和 DNA 损伤。

12) Zeng Ming et al. (2014): "The study indicates, 60-180 mg·L-1 concentration glyphosate causes obvious damage to GC-1 cells, its mechanism might be oxidative stress induced by gyphosate, leading to cell permeability increase and DNA damage."

13）赵文红等（2013）：草甘膦对小鼠 sertoli 细胞有一定的毒性,能诱导细胞凋亡及抑制细胞增殖,且随草甘膦剂量的增加,有害作用有增加的趋势;同时能抑制 ABP 和波形蛋白 mRNA 的表达。

13) Zhao Wenhong et al. (2013): "Conclusion: Glyphosate causes certain toxicity to mice sertoli cells, can induce cell apoptosis and inhibit cell proliferation, and the harm increases with glyphosate dosage increase; At the same time can inhibit the expression of ABP and wave shape protein mRNA."

1) 邬惠琼,草甘膦对大鼠细胞色素 P450 2B1 和 P450 2C11 基因表达的影响,《卫生毒理学杂志》1996 年第 10 卷第 4 期，231-234 页
（作者邬惠琼单位：武汉同济医科大学公共卫生学院环境毒理学研究室。）
1) Wu Hui-qiong, Glyphosate impact on rat cytochrome P450 2 B1 and P450 2 c11 gene expression, Health Toxicology Journal, 1996 10(4): 231-234 [Chinese]
(Organization: Environment Toxicology Research Section, Public Health College, Wuhan Tongji Medical University.)
http://www.cnki.com.cn/Article/CJFDTotal-WSDL604.004.htm

2) 耿德贵等，除草剂农达对黄鳝致突变性研究，《徐州师范大学学报(自然科学版)》 2000 年 02 期
2) Geng De-gui et al., Study of Herbicide Roundup impact on yellow eel mutagenic, Journal of Xuzhou Normal University (Natural Science Edition), 2000(2)
http://www.cnki.com.cn/Article/CJFDTotal-XZSX200002018.htm

3) 南旭阳，除草剂"草甘膦"对鲫鱼外周血红细胞微核及核异常的影响[J]. 安徽师范大学学报：自然科学版，2001，24(4)：329-331.
3) Nan Xu-yang, Impact of glyphosate herbicide on carp peripheral blood erythrocyte

micronucleus and nuclear anomalies, Journal of Anhui Normal University (Natural Science Edition), 2001,24(4): 329-331 [Chinese]
http://www.cqvip.com/qk/97138X/200006/4887295.html

4) 南旭阳，除草剂草甘膦对鲫鱼血细胞及血红蛋白影响的研究，甘肃科学学报，2002（4）
(就职机构：浙江温州师范学院生物与环境科学系)
(Organization: Biology & Environment Science Dept., Zhejiang Wenzhou Normal College)
4) Nan Xu-yang, Study of impact of glyphosate herbicide on carp blood cells and hemoglobin, Gansu Science, 2002(4)
http://www.cnki.com.cn/Article/CJFDTotal-GSKX200204015.htm

5) 南旭阳，张艳丹，黄小莲．除草剂"草甘膦"对泥鳅白细胞的影响[J]，温州师范学院 学报：自然科学版，2003,24(2):72-74 就职机构：温州师范学院生物与环境科学学院
5) Nan Xu-yang et al., Impact of glyphosate herbicide on loach white blood cells, Journal of Wenzhou Normal University: (Natural Science Edition), 2003,24(2): 72-74 [Chinese]
http://www.cnki.com.cn/Article/CJFDTotal-WZSF200302019.htm

6) 中国学者康菊芳 et al.，草甘膦对小鼠的致突变作用研究（《癌变.畸变.突变》，2008 年 03 期）
6) Kang Ju-fang et al., Study of glyphosate effect causing mutagenic on rats, Carcinogenesis, Teratogenesis & Mutagenesis, 2008(3)
http://www.cnki.com.cn/Article/CJFDTotal-ABJB200803018.htm

7) 王非，农达 41％草甘膦对人 L02 肝细胞损伤的研究（中南大学硕士论文）2008
7) Wang Fei, Study of Roundup 41% causing damage to human L02 liver cells, Master's thesis, Zhongnan University, 2008
http://cdmd.cnki.com.cn/article/cdmd-10533-2008165795.htm

8) 黄婷，农达对雄性生殖细胞的毒性作用及其机制的初步研究，中南大学（硕士论文），2010
8) Huang Ting, Preliminary study of Roundup's toxicity effect and mechanism on male reproductive cells, Master's thesis, Zhongnan University
http://cdmd.cnki.com.cn/Article/CDMD-10533-2010187394.htm
全文链接：http://www.doc88.com/p-974197814056.html

9) 李娇 et al.，8 种常见农药对海胆胚胎各发育期的急性毒性， 生态毒理学报，2010(2)
(机构：大连海洋大学、农业部海洋水产增养殖学与生物技术重点开放实验室)
(Organization: Marine Fisheries Aquaculture & Key Laboratory of Biotechnology, Dalian Marine University and Ministry of Agriculture)

9) Li Qiao, et al., Acute toxicity of eight types of pesticides to sea urchin embryos during different phases of development”.
http://d.wanfangdata.com.cn/Periodical_cyyhj201002014.aspx

10) 赵伟，曹曦予，吴艳萍，等. 草甘膦致小鼠机体氧化损伤作用的研究[J]。毒理学杂志，2011,25(5):364-366
10) Zhao Wei et al., Study of **oxidative damage of the body** caused by glyphosate, Toxiology Journal, 2011,25(5):364-366 [Chinese]
http://www.cnki.com.cn/Article/CJFDTotal-WSDL201105013.htm

11) 俞慧;江城梅;赵文红;草甘膦毒性作用研究进展[J];蚌埠医学院学报;2012 年 06 期
11) Yu Hui et al., Progress in study of glyphosate toxicity, 2012(6) [Chinese]
http://www.cnki.com.cn/Article/CJFDTOTAL-BANG201206050.htm
http://www.doc88.com/p-666125982792.html

12) 曾明、黄婷等，草甘膦对 GC-1 小鼠精原细胞的毒性作用及 N-乙酰半胱氨酸的干预效应，生态毒理学报，2014 年 01 期
12) Zeng Ming, Huang Ting et al., Glyphosate toxicity to mice GC-1 sperm cells and the interference effect of N-acetyl cysteine, Ecological Toxicology Bulletin, 2014(1) [Chinese]
http://www.cnki.com.cn/Article/CJFDTotal-STDL201401031.htm
【作者单位】: 中南大学公共卫生学院卫生毒理学系;长沙市疾病预防控制中心理化检验科;

Author's organization: Hygene Toxicology Dept., Public Health College, Zhongnan University; Changsha Disease Prevention & Control Center

【作者单位】： 蚌埠医学院预防医学系营养与食品卫生学教研室;蚌埠医学院第一附属医院麻醉科;

Author organization: Nutrition & Food Hygene hygiene section, Preventive Medicine Dept., Banpu Medical College; Anesthesia Dept. of No.1 Hospital attached to the Bangpu Medical College.

13) 赵文红、俞慧等，草甘膦对小鼠睾丸支持细胞凋亡及雄激素结合蛋白、波形蛋白 mRNA 表达的影响，**南方医科大学学报,** 2013 年 11 期

13) Zhao Wen-hong, Yu Hui et al., Glyphosate's effect supporting cell apoptosis and expression of androgen binding protein, wave shape protein mRNA, Southern Medical University Bulletine, 2013(11)

http://www.cnki.com.cn/Article/CJFDTotal-DYJD201311033.htm

附录 3：国外学者研究发现草甘膦或草甘膦除草剂农达致细胞毒性、致 DNA 损伤、致畸、致突变、致生殖毒性、致流产 46 项科学试验证据！

Attachment 3: Forty six studies by overseas scholars found that glyphosate or glyphosate formulated herbicides cause cell toxicity, DNA damage, teratogenic, mutagenic, and reproductive toxicity, along with miscarriage!

要点概述

Summary of Main Points

科学证据 1 Kale, P.G. et al.,（**1995 年）：草甘膦除草剂农达等九种除草剂与杀虫剂都发现在数种细胞中造成突变。**

1) Kale, P.G. et al, (1995): Roundup and others total nine herbicides and pesticides were tested for their mutagenicity using the Drosophila sex-linked recessive lethal mutation assay. These are Ambush, Treflan, Blazer, Roundup, 2,4-D Amine, Crossbow, Galecron, Pramitol, and Pondmaster. All chemicals induced significant numbers of mutations in at least one of the cell types tested.

科学证据 2 Yousef MI et al.（1995）：草甘膦造成实验兔体重、性欲、射精量、精子浓度等指标下降，危害精子质量的作用终止处理后继续发展，而且剂量依赖。机理可能是草甘膦对精子声称的直接细胞毒性，和/或间接通过控制繁殖效率的视丘下部垂体睾丸轴造成这些损害。

2) Yousef MI et al. (1995): Two sublethal doses of Carbofuran (carbamate insecticide) and Glyphosate (organophosphorus herbicide) treatment resulted in a decline in body weight, libido, ejaculate volume, sperm concentration, semen initial fructose and semen osmolality. This was accompanied with increases in the abnormal and dead sperm and semen methylene blue reduction time. The hazardous effect of these pesticides on semen quality continued during the recovery period, and was dose-dependent. These effects on sperm quality may be due to the direct cytotoxic effects of these pesticides on spermatogenesis and/or indirectly via hypothalami-pituitary-testis axis which control the reproductive efficiency.

科学证据 3 Savitz, D.A. et al.（**1997**）**：一些种类农药（阿特拉津、草甘膦、有机磷、**2,4-二氯苯氧乙酸与杀虫剂） **...基于这些数据，尽管对接触的评估有限，作者鼓励特别对配偶发生流产和早产的男性接触进一步评估。**

3) Savitz, D.A. et al (1997) A variety of chemicals (atrazine, glyphosate, organophosphates, 4-[2,4-dichlorophenoxy] butyric acid, and insecticides)... Based on these data, despite limitations in exposure assessment, the authors encourage continued evaluation of male exposures, particularly in relation to miscarriage and preterm delivery.

科学证据 4 Claudia Bolognesi et al.（**1997**）**：在该项研究中，配方制剂农达及其活性成分草甘膦，体内和体外试验中，对诱发 DNA 损伤与染色体的影响，采用同样的分析方法进行试验。... 在体内和体外试验中，观察到配方制剂农达及其活性成分草甘膦通过 DNA 单链断裂与 8-OHdG 造成的 DNA 损伤活动性与染色体改变显著增加。配方制剂显示基因毒性活动微弱增加。**

4）Claudia Bolognesi et al.（1997）:In this study, the formulated commercial product, Roundup, and its active agent, glyphosate, were tested in the same battery of assays for the induction of DNA damage and chromosomal effects in vivo and in vitro. ... A DNA-damaging activity as DNA single-strand breaks and 8-OHdG and a significant increase in chromosomal alterations were observed with both substances in vivo and in vitro. A weak increment of the genotoxic activity was evident using the technical formulation.

科学证据 5 Lioi, M.B. et al.（**1998**）**：对草甘膦、vinclozolin、阿特拉**

律进行了研究。在我们的实验条件下，试验的每种化学品产生了随剂量增加的
异常细胞与 SCE 细胞增加。

5）Lioi, M.B. et al. (1998)The pesticides gliphosate, vinclozolin, and atrazine have been studied ... In our experimental conditions, each chemical compound tested produced a dose-related increase in the percent of aberrant cells and an increase of SCE/cell.

科学证据 **6** Peluso M.（**1998**）：本研究表明，农达可以诱发小鼠中存在剂量依赖性的肝肾中 **DNA** 加合物的形成。在小鼠肝肾中观察到的农达相关 **DNA** 加合物在除草剂试验剂量观察到（600 mg/kg）各自为 3.0 +/- 0.1 (SE)与 1.7 +/- 0.1 **(SE)** 加合物**/10(8)** 核苷酸。农达造成的 **DNA** 加合物与其活性成分草甘膦无关，而与配方制剂农达中混合的另外一种不明成分有关。

6）Peluso M.,（1968）:Roundup is able to induce a dose-dependent formation of DNA adducts in the kidneys and liver of mice. The levels of Roundup-related DNA adducts observed in mouse kidneys and liver at the highest dose of herbicide tested (600 mg/kg) were 3.0 +/- 0.1 (SE) and 1.7 +/- 0.1 (SE) adducts/10(8) nucleotides, respectively. The Roundup DNA adducts were not related to the active ingredient, the isopropylammonium salt of glyphosate, but to another, unknown component of the herbicide mixture. Additional experiments are needed to identify the chemical specie(s) of Roundup mixture involved in DNA adduct formation.

科学证据 **7 Peggy J. Perkins at el.**（**2000**）：使用非洲爪蟾蜍晶胚致畸形作用 **FETAX** 分析，比较农达与其表明活性剂（**POEA**）的毒性，比较配方制剂农达（含表面活性剂 **POEA**）与 **Rodeo**（草甘膦除草剂，无表面活性剂 **POEA**）的毒性。对半致死量（**LC50**）进行比较表明配方制剂农达的毒性为 **Rodeo** 的 **700** 倍。对表面活性剂 **POEA** 对非洲爪蟾蜍尽管进行了有限的试验，每次试验显示 **POEA** 的半致死量

（LC50）浓度比农达或 Rodeo 的低，表明 POEA 的毒性更大。需要进行更多的试验，但是看来表面活性剂单独对配方制剂农达更大的毒性负责。这种更大的毒性看起来不大像是因为**晶胚摄入了更多草甘膦而强化。**

7) **Peggy J. Perkins at el.** (2000) Comparative toxicity of Roundupt and its surfactant polyoxyethyleneamine (POEA) to *Xenopus laevis*, and comparing Roundup formulation and the Rodeo formulation (with no surfactant POEA), using Frog Embryo Teratogenesis Assay—*Xenopus* (FETAX). A comparison of LC50 concentrations indicated that the Roundup formulation of glyphosate was 700 times as toxic as the Rodeo formulation. Even though a limited number of tests were performed to evaluate the effects of the surfactant POEA on *X. laevis*, each test showed a lower LC50 value for POEA alone than for either Roundup or Rodeo. More studies are needed, but it seems likely that the surfactant itself is responsible for the greater toxicity displayed by the Roundup formulation of glyphosate. It seems less likely that the greater toxicity is due to enhanced uptake of glyphosate by the embryos.

科学证据 8 Walsh, L.P. et al.**（2000 年）：结论，农达通过类固醇激素合成畸形调节蛋白（StAR protein）表达中后转录减少干扰睾丸间质细胞中类固醇生成。在设计内分泌干扰研究中用类固醇激素合成畸形调节蛋白作为端点值得进一步考虑。尽管农达减少类固醇激素合成，这种除草剂的活性成分并没有改变类固醇生成，表明农达配方中至少要包括另外一种成分来干扰类固醇激素合成。由于农达的配方专有保密，需要进一步研究来识别农达中的组分及其干扰类固醇激素合成的能力。**

8) Walsh, L.P. et al. (2000): In conclusion, Roundup disrupted steroidogenesis in Leydig cells through a post-transcriptional reduction in StAR protein expression. The use of StAR as an end point in studies concerning endocrine disruption merits further consideration. Although Roundup decreased steroidogenesis, the active ingredient of this herbicide, glyphosate, did not alter steroid production, indicating that at least one other component of the formulation is required to disrupt steroidogenesis. Because the formulation of Roundup is proprietary, further studies are needed to identify the components in Roundup and their ability to disrupt steroidogenesis.

科学证据 9 Daruich, J. et al.**（2001）：对怀孕鼠肝、心与脑脱氢酶活动的研究，结论草甘膦在雌鼠及其后代中造成不同种类的失调。**

9) Daruich, J. et al. (2001)We determined the effects of these compounds on the levels and activities of the P450scc enzyme (which converts cholesterol to pregnenolone) and the 3beta-hydroxysteroid dehydrogenase (3beta-HSD) enzyme (which converts pregnenolone to progesterone). Of the pesticides screened, only the pesticide Roundup inhibited dibutyryl [(Bu)(2)]cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants.

科学证据 10 T E Arbuckle et al.**（2001）：孕前接触草甘膦除草剂提高而后流产的风险，孕后接触草甘膦除草剂通常与晚期自然流产相关。**

10) T E Arbuckle et al. (2001) For late abortions, preconception exposure to glyphosate (OR = 1.7; 95% CI, 1.0-2.9), ... was associated with elevated risks. Postconception exposures were generally associated with late spontaneous abortions.

科学证据 11 Marc J et al.**（2002）：简要讲，农达通过草甘膦及其配方制剂的协同效应延迟 CDK1/细胞周期蛋白 B 的活动性影响细胞的生长周期。考虑到不同物种中 CDK1/细胞周期蛋白 B 调制器的普遍性，我们质疑草甘膦与农达对人类健康的安全性。**

11) Marc J et al. (2002): Roundup delayed the activation of CDK1/cyclin B in vivo. Roundup inhibited also the global protein

synthetic rate without preventing the accumulation of cyclin B. In summary, Roundup affects cell cycle regulation by delaying activation of the CDK1/cyclin B complex, by synergic effect of glyphosate and formulation products. Considering the universality among species of the CDK1/cyclin B regulator, our results question the safety of glyphosate and Roundup on human health.

科学证据 12 Dallegrave, E. et al.（**2003**）**：该项研究旨在评估草甘膦除草剂（在巴西商业化的）对** Wistar 大鼠的致畸形毒性。从孕期 6 到 15 天用草甘膦 500、750 或 1000 mg/kg 水溶液口服给怀孕鼠。结果显示用 1000 mg/kg 草甘膦处理的大鼠死亡率为 50%。与对照组相比，500、750 与 1000 mg/kg 草甘膦处理鼠胎儿中观察到 15.4%、33.1%、42.0%与 57.3%的胎儿中骨骼异常。我们可以结论草甘膦除草剂农达对大鼠诱发了胎儿骨骼发育迟缓。

12）Dallegrave, E. et al. (2003) The aim of this study was to assess the teratogenicity of the herbicide glyphosate-Roundup (as commercialized in Brazil) to Wistar rats. Dams were treated orally with water or 500, 750 or 1000 mg/kg glyphosate from day 6 to 15 of pregnancy. Results showed a 50%, mortality rate for dams treated with 1000 mg/kg glyphosate. Skeletal alterations were observed in 15.4, 33.1, 42.0 and 57.3% of fetuses from the control, 500, 750 and 1000 mg/kg glyphosate groups, respectively. We may conclude that glyphosate-Roundup is toxic to the dams and induces developmental retardation of the fetal skeleton.

科学证据 13 Lajmanovich RC et al.（**2003**）**：通过实验发现草甘膦配方除草剂在所有试验中对蝌蚪造成发育不良（颅面与嘴巴畸形、眼睛歪斜畸形、尾巴弯曲），而且随时间与浓度增加。**

13) Lajmanovich RC et al. (2003) Larval maldevelopment (craniofacial and mouth deformities, eye abnormalities and bent curved tails) occurred in all tests and increased with time and GLY-F

concentration.

科学证据 14 Cox, C.（**2004**）：**草甘膦已经显示有致癌作用。三项最近的研究发现接触草甘膦与非霍奇金淋巴瘤（一种癌症）的关联。**

14) Cox, C. (2004).Glyphosate has been shown to have carcinogenic effects. Three recent studies found a link between glyphosate exposure and non-Hodgkin's lymphoma (a type of cancer).

科学证据 15 Marc, J. et al.（**2004**）：**对不同厂家数种草甘膦除草剂干预细胞周期的能力进行比较。所有的草甘膦除草剂都诱发了细胞周期控制失效。**

15) Marc, J. et al. (2004): Several glyphosate-based pesticides from different manufacturers were assayed in comparison with Roundup 3plus for their ability to interfere with the cell cycle regulation. All the tested products, Amega, Cargly, Cosmic, and Roundup Biovert induced cell cycle dysfunction.

科学证据 16 Marc, J et al.（**2004 年**）：**草甘膦配方除草剂对细胞周期的影响在 DNA 响应检查点 S 阶段发挥作用。抑制 CDK1 / 15 B 细胞周期蛋白酪氨酸脱磷酸作用导致阻止 G2 / M 过渡和细胞周期的进展。**

16) Marc, J et al. (2004): We conclude that formulated glyphosate's effect on the cell cycle is exerted at the level of the DNA-response checkpoint of S phase. The resulting inhibition of CDK1/cyclin B Tyr 15 dephosphorylation leads to prevention of the G2/M transition and cell cycle progression.

科学证据 17 Benedettia, A.L. et al.（**2004**）：**该项研究的目的是分析草甘膦除草剂**-Biocarb（在巴西商业化的除草剂）对 Wister 大鼠的肝影响。... 我们结论，**草甘膦除草剂**-Biocarb 在实验模型中可能诱发肝组织学改变，以及肝泄漏出 AST 与 ALT 进入血清。

17) Benedettia, A.L. et al., (2004) The object of this study was to analyze the hepatic effects of the herbicide Glyphosate-Biocarb (as commercialized in Brazil) in Wistar rats. ... We may conclude that Glyphosate-Biocarb may induce hepatic histological changes as well as AST and ALT leaking from liver to serum in experimental models.

科学证据 18 John F Acquavella et al.（**2004**）**：孟山都公司的报告：喷洒草甘膦除草剂的当天，60%的男性农民尿中检测到草甘膦，几何平均数农达为 3 ppb，最高值为 233 ppb，最高估计系统性剂量为 0.004 mg/kg。他们的配偶中 4%的女性尿中检测到草甘膦，最高值为 3 ppb；他们的孩子尿样中 12%在喷洒草甘膦除草剂的当天检测到草甘膦，最高浓度为 29 ppb。**

18）John F Acquavella et al. (2004): This study by Monsanto reported: Sixty percent of farmers had detectable levels of glyphosate in their urine on the day of application. The geometric mean (GM) concentration was 3 ppb, the maximum value was 233 ppb, and the highest estimated systemic dose was 0.004 mg/kg. For spouses, 4% had detectable levels in their urine on the day of application. Their maximum value was 3 ppb. For children, 12% had detectable glyphosate in their urine on the day of application, with a maximum concentration of 29 ppb.

科学证据 19 Marc, J. et al.（**2005 年**）**：在海胆中观察到草甘膦除草剂农达中表面活性剂 POEA 致显著毒性。**

19）Marc, J. et al.（2005）：The surfactant polyoxyethylene amine (POEA), the major component of commercial Roundup, was found to be highly toxic to the embryos when tested alone and therefore could contribute to the inhibition of hatching.

科学证据 20 Lajmanovich, R.C. et al.（**2005**）**：草甘膦配方除草剂在所有试验中对蝌蚪造成发育不良（颅面与嘴巴畸形、眼睛歪斜畸形、尾**

巴弯曲），而且随时间与浓度增加。... 在 3.07 mg/L 浓度暴露 1 天的畸形最少，暴露于 7.5 mg/L 浓度时则 90%畸形。该试验确认草甘膦除草剂对蝌蚪的致畸形作用。

20) Lajmanovich, R.C. et al. (2005): Larval maldevelopment (craniofacial and mouth deformities, eye abnormalities and bent curved tails) occurred in all tests and increased with time and GLY-F conentration. ... Malformation were minimal at 3.07 mg/L exposed for one day, whereas greater that 90% were malformed at a GLY-F level of 7.5 mg/L. The current test confirmed the malformation effects of GLY-F on tadpoles.

科学证据 21（2005）：研究试验口服 1%浓度草甘膦在 21 天孕期中对怀孕鼠的血清与肝及其胎儿的脂质过氧化与抗氧化酶系统的影响。结果提议，摄入草甘膦诱发过量脂质过氧化，导致对怀孕鼠及其胎儿抗氧化防御系统过量。

21) **Beuret CJ et al.**The present study has investigated the effects that 1% glyphosate oral exposure has on lipoperoxidation and antioxidant enzyme systems in the maternal serum and liver of pregnant rats and their term fetuses at 21 days of gestation. The results suggest that excessive lipid peroxidation induced with glyphosate ingestion leads to an overload of maternal and fetal antioxidant defense systems.

科学证据 22 Richard S et al.（2005）：研究显示草甘膦在低于农业使用浓度条件下在 18 小时内对人类胎盘 JEG3 细胞有毒性，毒性作用随浓度与时间或者存在农达辅佐剂时增加。农达总是比其活性成分草甘膦更为毒性。草甘膦除草剂干扰芳香化酶活性与 mRNA 水平，并与纯化酶的活性部位相互作用，但是农达配方在微粒体或细胞培养中加强了草甘膦的毒性作用。**我们认为农达配方中辅佐剂的存在强化了的草甘膦的生物可获得性和/或生物积蓄。**

22) **Richard S et al. (2005):** We show that glyphosate is toxic to human placental JEG3 cells within 18 hr with concentrations lower than

those found with agricultural use, and this effect increases with concentration and time or in the presence of Roundup adjuvants. Surprisingly, Roundup is always more toxic than its active ingredient. The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation in microsomes or in cell culture. We conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. We suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

科学证据 **23** Sparling DW **et al.（2006）：孵化成功率在接触最高浓度时显著低于（73%）其他处理（80-100%）。基因损伤，由流式细胞术测量，随浓度增高而增加，最高剂量例外。还存在着乌龟晶胚的健康可能以本研究没有检测到的方式受到草甘膦影响的风险。**

23) Sparling DW **et al. (2006):** Hatching success at the highest concentration was significantly lower (73%) than in other treatments (80-100%). ... Genetic damage, as measured by flow cytometry, increased with treatment concentration except for the highest dose. ... There also is a risk that the health of turtle embryos may be affected in ways not measured in the present study.

科学证据 **24 Oliveira AG et al.（2007）：接触草甘膦除草剂造成对睾丸与附睾区域构造的改变，还改不了血清中睾丸激素与雌二醇的水平，改变了睾丸雄性激素受体的表达。更明显的近端传对微胆管和附睾导管造成有害影响，表明雄生殖器中这些部位对草甘膦更为敏感。造成的影响具有剂量依赖性，表明草甘膦除草剂可能在动物雄生殖系统中造成形态生理学失调。**

24) **Oliveira AG et al.** (2007): The exposure to the herbicide resulted in alterations in the structure of the testis and epididymal region as well as in the serum levels of testosterone and estradiol, with changes

in the expression of androgen receptors restricted to the testis. The harmful effects were more conspicuous in the proximal efferent ductules and epididymal ducts, suggesting higher sensitivity of these segments among the male genital organs. The effects were mostly dose dependent, indicating that this herbicide may cause disorder in the morphophysiology of the male genital system of animals.

科学证据 **25** César Paz-y-Miño et al.（**2007**）：对厄瓜多尔北部飞机喷洒添加表面活性剂的草甘膦除草剂的后果。**...** 结果显示，与对照组（彗星长度=**25.94 μm**）相比，暴露组中更高程度的 **DNA** 损伤（彗星长度=**35.5 μm**）。这些结果提议飞机喷洒的草甘膦除草剂配方对接触的人士致基因毒性，造成 **DNA** 损伤。

25) César Paz-y-Miño et al. (2007): We analyzed the consequences of aerial spraying with glyphosate added to a surfactant solution in the northern part of Ecuador. ... The results showed a higher degree of DNA damage in the exposed group (comet length = 35.5 μm) compared to the control group (comet length = 25.94 μm). These results suggest that in the formulation used during aerial spraying glyphosate had a genotoxic effect on the exposed individuals.

科学证据 **26 Bellé R et al.**（**2007**）：癌症生物学中新的见解导致对于癌发生的最初起源两种基本概念。首先，癌症是 **DNA** 损伤检查点功能障碍的结果，而且癌症看来是正常干细胞（**NCS**）转变为癌症干细胞（**CSC**）的结果。第二个方面概念对“癌症”提议了新的定义，因为癌症干细胞（**CSC**）可以在出现任何临床证据前探测到。因为早期发育从受精卵开始，它是一种初级的干细胞，海胆早期发育允许对癌症化过程早期步骤进行分析。在毒理学以及人类健康发生率领域，海胆实验模型允许在任何流行病学证据出现前从单独或结合分子对癌症风险进行评估。用海胆晶胚对世界广泛使用的含活性成分草甘膦的除草剂配方农达进行了试验；它表明激活细胞头一个发展周期的 **DNA** 损伤检查点。

26) **Bellé R et al. (2007):** New insights in cancer biology lead to two fundamental concepts about the very first origin of cancerogenesis. Cancers result from dysfunction of DNA-damaged checkpoints and cancers appear as a result of normal stem cell (NCS) transformation into a cancer stem cell (CSC). The second aspect suggests a new definition of "cancer", since CSC can be detected well before any clinical evidence. Since early development starts from the zygote, which is a primary stem cell, sea urchin early development allows analysis of the early steps of the cancerization process. In the field of toxicology and incidence on human health, the sea urchin experimental model allows assessment of cancer risk from single or combined molecules long before any epidemiologic evidence is available. Sea urchin embryos were used to test the worldwide used pesticide Roundup that contains glyphosate as the active herbicide agent; it was shown to activate the DNA-damage checkpoint of the first cell cycle of development.

科学证据 27 Dallegrave E et al.（**2007**）：研究结果表明，草甘膦除草剂农达没有诱发雌鼠毒性，但是对雄性后代造成了有害繁殖性影响：鼠仔成年后每个附睾精子数量与每天精子产生量减少、增加异常精子百分比，进入青春期后血清睾丸激素水平产生与草甘膦接触量相关减少，同时在两个阶段出现精子退化迹象。后代雌鼠仔长大后仅发生阴道开通延迟。这些发现表明，子宫内与哺乳期接触草甘膦除草剂农达可能对雄鼠仔进入青春期与成年期阶段的生殖系统诱发显著有害影响。

27) Dallegrave E et al. (2007) The results showed that glyphosate-Roundup did not induce maternal toxicity but induced adverse reproductive effects on male offspring rats: a decrease in sperm number per epididymis tail and in daily sperm production during adulthood, an increase in the percentage of abnormal sperms and a dose-related decrease in the serum testosterone level at puberty, and signs of

individual spermatid degeneration during both periods. There was only a vaginal canal-opening delay in the exposed female offspring. These findings suggest that in utero and lactational exposure to glyphosate-Roundup may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood.

科学证据 28 McComb et al.（**2007**）**：试管试验表明，草甘膦对试验鼠肝脏细胞中的线粒体发挥一种氧化磷酸化解耦联剂作用。**

28) McComb et al. **(2007):** In *in vitro* tests found that glyphosate acts in the mitochondria of the rat liver cells as an oxidative phosphorylation decoupling agent.

科学证据 29 Soso AB **et al.**（**2007**）**：结果表明水中存在着的草甘膦危害 Rhamdia quelen 的繁殖、改变类固醇特性与卵的活力。**

29) Soso AB **et al. (2007)** The results indicate that the presence of glyphosate in water was deleterious to *Rhamdia quelen* reproduction, altering steroid profiles and egg viability.

科学证据 30 Hokanson R **et al.**（**2007**）**：最为普遍使用的商业性与家用化学品为草甘膦除草剂。尽管普遍认为草甘膦相对无毒性，我们对这种化学品做体内实验微阵列分析，来评价草甘膦改变人类细胞多种基因表达的能力。我们选择了一组基因，由 DNA 微阵列分析确定其特异表达，使用定量性实时 PCR 来证实表达改变的状态。我们讨论对这些基因报告的功能，强调可能在成年人或者子宫内暴露于草甘膦的胚胎中可能启动不良健康影响的改变了的生理学状态。**

30) Hokanson R **et al. (2007) Among the chemicals most commonly used, both commercially and in the home, is the herbicide glyphosate. Although glyphosate is commonly considered to be relatively non-toxic, we utilized *in vitro* DNA microarray analysis of this chemical to evaluate its capacity to alter the expression of a variety of genes in human cells. We selected a group of genes,**

determined by DNA microarray analysis to be dysregulated, and used quantitative real-time PCR to corroborate their altered states of expression. We discussed the reported function of those genes, with emphasis on altered physiological states that are capable of initiating adverse health effects that might be anticipated if gene expression were significantly altered in either adults or embryos exposed *in utero*.

科学证据 **31** Mañas F et al.（**2009**）：**AMPA** 是草甘膦的主要环境性降解物。**...** 在彗星试验中，与对照组相比，细胞 **2.5-7.5mM** 水平 **DNA** 损伤显示显著增加。与对照组相比，我们发现人类淋巴细胞中在 **1.8mM** 水平 **AMPA** 造成的染色体断裂效果统计显著。在活体试验中，在 **200-400mg/kg** 水平，微核测试统计呈现明显增加。**AMPA** 在进行的三项试验中皆致细胞毒性。

31) Mañas F et al. (2009): AMPA is the major environmental breakdown product of glyphosate. The purpose of this study is to evaluate the in vitro genotoxicity of AMPA using the Comet assay in Hep-2 cells after 4h of incubation and the chromosome aberration (CA) test in human lymphocytes after 48h of exposition. Potential in vivo genotoxicity was evaluated through the micronucleus test in mice. In the Comet assay, the level of DNA damage in exposed cells at 2.5-7.5mM showed a significant increase compared with the control group. In human lymphocytes we found statistically significant clastogenic effect AMPA at 1.8mM compared with the control group. In vivo, the micronucleus test rendered significant statistical increases at 200-400mg/kg. AMPA was genotoxic in the three performed tests.

科学证据 **32** Nora Benachour and Gilles-Eric Séralini（**2009**）：对草甘膦及其主要代谢物 **AMPA**，以及草甘膦添加除草剂配方中主要辅佐剂 **POEA**（表面活性剂，稀释 **10** 万倍，对三种不同的人类细胞的毒性。这样的稀释水平，远远低于农业应用推荐的水平，并对应于食品

或者饲料中草甘膦残留的低水平。三种人类细胞分别为新生儿脐带静脉的细胞、293 胚肾细胞与 JEG3 胎盘细胞系。所有草甘膦的配方在 24 小时内造成所有细胞死亡，通过抑制线粒体琥珀酸脱氢酶活性，以及通过释放胞质膜伤害腺苷酸激酶测量膜损伤导致坏疽。通过激化酶的半胱天冬酶 3/7 活性诱发细胞凋亡。单独草甘膦激起仅细胞凋亡，而人脐静脉内皮细胞（HUVEC）在这个水平上 100 倍更敏感。有害效应与草甘膦浓度不成比例，更加取决于除草剂辅佐剂的性质。草甘膦代谢物 AMPA 与 POEA（草甘膦除草剂配方中的表面活性剂），分别单独作用或者合在一起协作用时，像草甘膦一样，损伤细胞膜，但是各自在不同的浓度发挥作用。它们与草甘膦一起的混合物的危害通常更强。结论，像 POEA 这样的辅佐剂，改变人类细胞的渗透性，以凋亡与坏疽方式强化诱发草甘膦诱发的毒性。草甘膦真正的阈值，必须考虑存在的辅佐剂，还必须考虑草甘膦的代谢、时间放大效应或生物蓄积作用。市场上销售的草甘膦除草剂，即便在抗草甘膦作物加工的食品与饲料残留水平，能够造成细胞损伤以至死亡。

32) Nora Benachour and Gilles-Eric Séralini (2009): We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup (R) formulations, from 10^5 times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only

apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation. This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

科学证据 33 Mariana, A. et al.**（2009）：我们研究了注射服用 1/50 与 1/250 半致死量（LD50）乐果、草甘膦、代森锌后，单独或者结合在一起，对大鼠的慢性作用。进行试验的这些农业化学品，增加了血清、肝脏与睾丸中氧化应激状态，并改变了生殖功能中的激素指标。氧化应激状态与遭受损伤的生物标记物水平，以及对抗氧化防御系统造成的改变减少了农药处理大鼠血清中睾丸激素、FSH（促卵泡生成 激素 ）与 LH（黄体生成 激素 ）水平。**

33) Mariana, A. et al. (2009): We studied the effect of chronic pesticide exposure in rats injected i.p. for 5 weeks with doses between 1/50 and 1/250 LD50 of dimethoate, glyphosate and zineb, either alone or in combination. All tested agrochemicals increased the oxidative stress status in the plasma, liver, and testes, and also modified hormonal parameters involved in reproductive function. The increase in oxidative stress and damage biomarker levels, as well as the alteration of the antioxidant defence system decreased testosterone, FSH and LH levels in

the plasma of pesticide-treated rats.

科学证据 **34** Mañas F et al.（**2009**）**：该研究中，彗星试验表明 400mg/kg 剂量草甘膦对小鼠 HEp-2 细胞与 MNT 试验中致成细胞毒性。... 结果显示这些酶活动性增加。依据所获得的结果，我们不能排除氧化应激成为潜在细胞毒性机制的可能。由于结果还不是结论性的，我们认为有必要对氧化应激与基因损伤之间可能的关联进一步研究。**

34) Mañas F et al. (2009): In the present study glyphosate was genotoxic in the comet assay in Hep-2 cells and in the MNT test at 400 mg/kg in mice. ... The results showed an increase in these enzyme activities. According to the obtained results we cannot discard the oxidative stress as a potential genotoxicity mechanism. Due to the fact that the results were not conclusive we believe it is necessary to carry on researching the possible connection between oxidative stress an genetic damage.

科学证据 **35 Prasad S et al.（2009）：和载体对照相比，两种草甘膦处理处理与时间方面相比，诱发 CA（染色体畸变）与 MN （微核）显著**（P < .05）**增加。细胞有丝分裂指数（MI）显著减少，表明草甘膦的细胞毒性很明显。该项研究的结果表明草甘膦对小鼠骨髓致染色体断裂与细胞毒性。**

35) **Prasad S et al.** (2009)：Glyphosate treatment significantly increases CAs (chromosomal aberrations) and MN (micronuclei) induction at both treatments and time compared with the vehicle control ($P < .05$). The cytotoxic effects of glyphosate were also evident, as observed by significant decrease in mitotic index (MI). The present results indicate that glyphosate is clastogenic and cytotoxic to mouse bone marrow.

科学证据 **36** Robin Mesnage et al.（**2009**）**：2009 年 1 月，一对农场夫妇与我们联系，因为他们三个孩子中有两位出生时有先天性缺陷。一**

个孩子有生长激素生长缺陷、还有一个出生时肛门闭锁、一个小心房中隔缺损。另外一个孩子遭受尿道下裂、有一个小阴茎、生长激素完全缺少，肛门也闭锁。所有这些缺陷发生在同一个孩子或者同一个家庭及其稀少。然而，某些这样的病状与其他症状一起确诊为斯特拉顿帕克综合征，其病因至今未知。他们明显与我们研究的出生缺陷重叠。到目前为止只有男性受此影响，而且所有病例零星发生，某些茁壮因而认为是 x 连锁隐性遗传。由于缺乏家族先例资料，而且到目前为止缺乏遗传来源，可以对环境根源假说进行探讨。怀孕前后，这个家庭使用了许多农药。丈夫每年喷洒 1.3 吨农药，其中包括 300 公升草甘膦除草剂，而且没有戴防护面具。这些农药中有多种已知是内分泌干扰剂的成分，如多菌灵、2,4-叶橙剂酸、草甘膦、碘苯腈、利谷隆，氟乐灵和烯菌酮。全家与父亲接触密切，吃他们花园的农产品，也可以通过喂养自己农场作物饲料的猪与家禽接触这些农药。

36) Robin Mesnage et al. (2009): In January 2009, a farming couple contacted us because two of their three children were born with congenital malformations. One had a somatotropic deficiency, an imperforate anus and a small atrial septal defect at birth. Another was suffering from hypospadias, had a micropenis, a total deficiency of growth hormone and presented also an imperforate anus. All these disorders are rarely encountered in the same person or family. Yet, in some cases these symptoms with others have been grouped under the Stratton-Parker syndrome, whose etiology remains unknown. They noticeably overlap our cases. Only males are affected up to date and all cases occurred sporadically, some authors have therefore proposed an X-linked recessive inheritance. Due to the absence of known familial antecedents, and lack of genetic origins evidenced to date, the hypothesis of an environmental origin can be explored. In particular, many pesticides were used by this family around pregnancies. The father sprayed, without protection, more than 1.3 tons of pesticides per year including 300 liters of glyphosate based herbicides. Among them are well-known endocrine disruptors such as carbendazim, 2,4-Dichlorophenoxyacetic acid,

glyphosate, ioxynil, linuron, trifluralin and vinclozolin. The whole family had close contact with the father, consumes products of their garden and can be exposed through the consumption of pigs and poultry fed with the farm harvest.

科学证据 37 Gasnier C et al.**（2009）：草甘膦为基础草甘膦在世界上最广泛使用。它们的残留成为环境中经常有的污染物。此外，这些除草剂还喷洒到最大量食用的转基因作物，这样的作物使其容忍细胞中高水平的这些成分。某些饲料中允许它们高达 400 ppm 残留。人类肝脏 HepG2 细胞是研究异型生物质毒性的知名模型，我们让人类肝脏 HepG2 细胞接触草甘膦及其四种不同配方除草剂制剂。通常仅在慢性活体内对单独草甘膦成分进行试验。我们用三种试验方法（Alamar Blue, MTT, ToxiLight），以及基因毒性（彗星试验）、抗雌激素（对 ERalpha, ERbeta）与抗雄激素效果（对 AR）做基因检测试验。我们还用芳香化酶活性与 mRNA 检测雄激素雌激素转换。所有指标在 24 小时内都受到亚农业用剂量用的草甘膦及其四种配方除草剂制剂所有成分的干扰。其效果更依赖于草甘膦除草剂的配方而非草甘膦的剂量。首先观察到的人类细胞内分泌干扰是最为活性配方制剂（R400）从 0.5 ppm 剂量在 MDA-MB453-kb2 细胞中对雄激素受体的作用，然后从 2 ppm 剂量起，HepG2 细胞的两个雌激素受体的转录活动性遭到抑制。从 10 ppm 剂量起，芳香化酶转录和活动收到干扰。在 Alamar Blue 试验（最敏感的）中，从 10 ppm 剂量其发生细胞毒性作用，但从 5 ppm 起发生 DNA 损伤。因此必须考虑食物、饲料或者环境中草甘膦除草剂对真实细胞的影响，对草甘膦分类为致癌物/致突变/致生殖毒性进行了讨论。**

37) Gasnier C et al. (2009): Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human

liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), plus genotoxicity (comet assay), anti-estrogenic (on ERalpha, ERbeta) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/ mutagens/reprotoxics is discussed.

科学证据 38 Romano RM et al.（2010）：研究结果表明商业配方的草甘膦除草剂在体内是一种威力强大的内分泌干扰剂，青春期接触时对老鼠的生育系统发育造成干扰。

38) Romano RM et al. (2010): These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.

科学证据 39 Jayawardene, U.A et al.**（2010）：用 1ppm 浓度，草甘膦对沙漏树蛙蝌蚪处理后，造成几种农药中最高的 69%畸形率。观察到的畸形主要为脊柱畸形，如驼背（驼背）和曲率（脊柱侧弯），也**

观察到而水肿和皮肤溃疡。

39) Jayawardene, U.A et al. (2010): Glyphosate recorded the highest percentage of malformation (69%) compared to other pesticides in 1.00 ppm concentration. Malformations observed were mainly in the spine, such as hunched back (kyphosis) and curvature (scoliosis), while edema and skin ulcers were also observed

科学证据 **40** Paganelli, A.et al.（**2010**）：非洲爪蟾蜍晶胚与稀释 **5000** 倍草甘膦除草剂一起孵化。经处理的晶胚高度异常，头盖与神经嵴明显改变，前后轴缩短。改变后神经嵴标记与颅软骨蝌蚪阶段畸形一致。注射单独草甘膦显示非常类似的畸形。草甘膦除草剂在鸡晶胚中显示类似的影响，显示一个逐渐失去了菱域。减少视觉囊泡和小头畸形。这表明草甘膦自己对观察到的畸形负责，而不是草甘膦除草剂配方中的表面活性剂或其他组分。一个报告基因分析，揭示草甘膦除草剂处理增加了非洲爪蟾蜍晶胚中的视黄酸活性，而且与 **RA** 拮抗剂的协作处理保持了草甘膦除草剂的致畸效应。因此结论草甘膦除草剂产生的显型主要是内源性类活动增加的结果。

40）Paganelli, A.et al. (2010) Xenopus laevis embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior−posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in Xenopus embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the

GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity.

科学证据 41 Rick A. Relyea（**2012**）：**美国匹兹堡大学生物科学教授，匹兹堡生态谐调实验室主任瑞克·利莱伊，发现农达在蝌蚪中诱发形态改变。在木蛙鱼豹蛙蝌蚪中，农达诱发与蜻蜓线索中诱发的相同方向与相同程度诱发了相对更深的尾部形态改变。根据利莱伊教授，这是表明一种除草剂可以诱发脊椎动物形态改变的头一项研究。此外，这些数据提议，农达可能激活了蝌蚪对抗天敌的发育性途径。合在一起，这些发现提议世界使用最为广泛的这种除草剂或许有过去尚未考虑过的对非靶标物质的更广泛作用。**

41) Rick A. Relyea (2012) Even more striking was the discovery that Roundup induced morphological changes in the tadpoles. In wood frog and leopard frog tadpoles, Roundup induced relatively deeper tails in the same direction and of the same magnitude as the adaptive changes induced by dragonfly cues. To my knowledge, this is the first study to show that a pesticide can induce morphological changes in a vertebrate. Moreover, the data suggest that the herbicide might be activating the tadpoles' developmental pathways used for antipredator responses. Collectively, these discoveries suggest that the world's most widely applied herbicide may have much further-reaching effects on nontarget species than previous considered.

科学证据 42 Fabio Leonardo Meza-Joya et al.（**2012**）：**草甘膦应用浓度超过 5.4 μg a.e./cm^2（体内）以及 95 μg a.e./mL 以上（试管内）显示细胞毒性清楚证据。青蛙（*Eleutherodactylus johnstonei*）试管内接触草甘膦配方，以剂量依赖方式诱导 DNA 破坏，在试验的所有剂量下统计显著（$P < 0.05$）。DNA 破坏随接触时间延长而增加，而后减少，表明无论体内还是试管内接触都发生 DNA 修理事件。**

42) Fabio Leonardo Meza-Joya et al. (2012): Glyphosate formulation at application rates above 5.4 μg a.e./cm^2 (in vivo) and

concentrations above 95 μg a.e./mL (in vitro) showed clear evidence of cytotoxicity. In vivo and in vitro exposure of *E. johnstonei*e rythrocytes to the glyphosate formulation induced DNA breaks in a dose-dependent manner with statistically significant values ($P < 0.05$) at all doses tested. DNA damage initially increased with the duration of exposure and then decreased, suggesting that DNA repair events were occurring during in vivo and in vitro exposures.

科学证据 43 Koller VJ et al.**（2012）：草甘膦除草剂是世界最大量销售的除草剂；最普遍的配方制剂农达含 POEA 作为其主要表面活性剂。最新的发现表明，暴露于草甘膦对人类可能造成 DNA 损伤与癌症。农达在>40mg/l 浓度接触 20 分钟后诱发急性细胞毒性，这是细胞膜与线粒体功能遭到损伤的结果。单细胞凝胶电泳分析表明，草甘膦与农达，> 20mg/l 浓度下，诱发 DNA 迁移。此外，**
...与早期的草甘膦对内部器官淋巴与细胞的研究进行比较表明，上皮细胞更容易受到细胞毒性，而且 DNA 损伤的性质与除草剂及其配方相关。由于我们发现农业中喷洒剂量稀释 450 倍的短期暴露造成基因毒性的影响，我们的发现表明，喷洒草甘膦除草剂可能造成吸入人类 DNA 损伤。此外，观察到反映 DNA 损伤的核畸变增加。接触 10-20mg/l 浓度 20 分钟后，微核和核味蕾的频率升高，而染色体桥（NPBs）仅在最高剂量（20 mg/l）增强。农达在所有条件下比起活性成分草甘膦更为活性。与先前对淋巴细胞和内部器官细胞的研究结果比较，除草剂及其配方，表明上皮细胞更容易致细胞毒性和 DNA 损伤。由于我们发现，在对应于农业喷洒浓度稀释 450 倍短暂接触后造成基因毒性效应相，我们的研究结果表明，吸入可能对暴露的个人造成 DNA 损伤。

43) Koller VJ et al. (2012): Glyphosate (G) is the largest selling herbicide worldwide; the most common formulations (Roundup, R) contain polyoxyethyleneamine as main surfactant. Recent findings indicate that G exposure may cause DNA damage and cancer in humans. R induced acute cytotoxic effects at concentrations >40 mg/l after 20 min,

which were due to membrane damage and impairment of mitochondrial functions. Both G and R induced DNA migration in single-cell gel electrophoresis assays at doses >20 mg/l. Furthermore, an increase of nuclear aberrations that reflect DNA damage was observed. The frequencies of micronuclei and nuclear buds were elevated after 20-min exposure to 10–20 mg/l, while nucleoplasmatic bridges were only enhanced by R at the highest dose (20 mg/l). R was under all conditions more active than its active principle (G). Comparisons with results of earlier studies with lymphocytes and cells from internal organs indicate that epithelial cells are more susceptible to the cytotoxic and DNA-damaging properties of the herbicide and its formulation. Since we found genotoxic effects after short exposure to concentrations that correspond to a 450-fold dilution of spraying used in agriculture, our findings indicate that inhalation may cause DNA damage in exposed individuals.

科学证据 44 Vandenberg LN **et al.（2012）：传统毒理学一直坚持的法则。近几十年，内分泌干扰化学品（EDC）的研究对毒理学传统概念"剂量致毒性"的法则提出了挑战，因为内分泌干扰化学品（EDC）高剂量的影响无法预测低剂量的影响。我们审查了内分泌干扰化学品（EDC）研究中的两项主要概念：低剂量与非单调性（nonmonotonicity）。... 我们结论，非单调性剂量响应曲线发生时，低剂量时的效应无法由高剂量观察到的效应进行预测。因此，对毒理学中化学测试与毒性确定需要做实质性改变来保护人类安全。**

44) Vandenberg LN **et al. (2012):** For decades, studies of endocrine-disrupting chemicals (EDCs) have challenged traditional concepts in toxicology, in particular the dogma of "the dose makes the poison," because EDCs can have effects at low doses that are not predicted by effects at higher doses. Here, we review two major concepts in EDC studies: low dose and nonmonotonicity. ... We conclude that when nonmonotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. Thus, fundamental changes in chemical testing and safety determination are needed to protect human health.

科学证据 45 Benedetti D et al.**（2013）：巴西** Rio Grande do Sul 州广泛种植（转基因）大豆，特别 Espumoso 市地区。这个区域大豆农工越来越多暴露于杀真菌剂、除草剂与杀虫剂。... 彗星分析与 BMCyt（微核和核芽）数据揭示大豆农工中的 DNA 损伤。还观察到细胞死亡（染色质浓缩、、核碎裂与核溶解细胞）。对口腔样本的微量元素含量进行了分析粒子激发 X 射线荧光分析。在工人的细胞中观察到了更高浓度的镁、铝、硅、磷、硫和氯。没有观察到(这两种问题)与使用个人防护设备、性别或农药的施用方式有任何联系。我们的研究结果表明应当监测大豆农场工人施用农药的基因毒害。

45) Benedetti D et al. (2013): Soybean cultivation is widespread in the State of Rio Grande do Sul (RS, Brazil), especially in the city of Espumoso. Soybean workers in this region are increasingly exposed to a wide combination of chemical agents present in formulations of fungicides, herbicides, and insecticides. ... Comet assay and BMCyt (micronuclei and nuclear buds) data revealed DNA damage in soybean workers. Cell death was also observed (condensed chromatin, karyorhectic, and karyolitic cells). Inhibition of non-specific choline esterase (BchE) was not observed in the workers. The trace element contents of buccal samples were analyzed by Particle-Induced X-ray Emission (PIXE). Higher concentrations of Mg, Al, Si, P, S, and Cl were observed in cells from workers. No associations with use of personal protective equipment, gender, or mode of application of pesticides were observed. Our findings indicate the advisability of monitoring genetic toxicity in soybean farm workers exposed to pesticides.

科学证据 46 Thongprakaisang S **et al.（2013）：草甘膦是最为广泛使用并被人们相信比其他农药毒性较低的除草剂的活性成分。然而，最近**

几项研究显示它对人类潜在危害健康，而且可能是一种内分泌干扰剂。该项研究集中于纯草甘膦对雌激素受体蛋白（**estrogen receptors**）促进的转录活动性及其表达。草甘膦，在 **10^{-12}** 至 **10^{-6} M**（万亿分之一至百万分之一质量）范围，在雌激素撤走状态下仅对人类激素-依赖乳房癌细胞 **T47D** 细胞发挥增殖作用，而对激素-独立乳房癌细胞 **MDA-MB231** 细胞系没有这种作用。诱发 **ERE**（雌激素反应元素）转录活动性达到 **T47D-KBluc** 细胞中对照组的 **5-13** 倍的产生增殖作用剂量的草甘膦，受到一种雌激素对抗药 **ICI 182780** 的抑制，表明草甘膦的雌激素活动性通过雌激素受体蛋白（**estrogen receptors**）发挥作用。此外，草甘膦既改变雌激素受体蛋白（**estrogen receptors**）**α**，有改变其 **β** 表达。这些结果显示，在低的与环境性相关浓度下，草甘膦具有雌激素性活动。草甘膦为基础的除草剂广泛用于（转基因）大豆种植，而我们的结果发现，存在着草甘膦与大豆中的一种植物雌激素染料木黄酮（**genistein**）之间的额外雌激素作用。然而，草甘膦对于（转基因）大豆的这种额外的作用需要进行进一步的动物试验。

46) Thongprakaisang S **et al. (2013):** Glyphosate is an active ingredient of the most widely used herbicide and it is believed to be less toxic than other pesticides. However, several recent studies showed its potential adverse health effects to humans as it may be an endocrine disruptor. This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10^{-12} to 10^{-6} M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity. Glyphosate-based herbicides are widely used for soybean cultivation, and our results also found that there was an additive estrogenic effect between glyphosate and genistein, a phytoestrogen in soybeans. However, these additive effects of glyphosate contamination in soybeans need further animal study.

1) Kale, P.G. et al, (1995). Mutagenicity testing of nine herbicides and pesticides currently used in agriculture. Environmental and Molecular Mutagenesis, 25, 148-153.

Kale, P.G. et al, (1995)。农业中目前使用的九种除草剂和杀虫剂的诱变性试验。环境与分子变异发生，25, 148-153.

Department of Biology, Alabama A. & M. University, Normal 35762, USA.

就职机构：阿拉巴马大学，生物系

http://www.ncbi.nlm.nih.gov/pubmed/7698107

2) Yousef MI et al., Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. J Environ Sci Health B. 1995 Jul;30(4):513-34.

Yousef MI et al.，呋喃丹与草甘膦对兔子精子特征的毒性影响，环境科学健康学报。1995 年 7 月，30(4):513-34.

就职机构：埃及亚历山大大学环境研究系

http://www.ncbi.nlm.nih.gov/pubmed/7797819

3) Savitz, D.A. et al, (1997). Male pesticide exposure and pregnancy outcome. American Journal of Epidemiology, 146, 1025-1035.

Savitz, D.A. et al, (1997)。男性接触农药及其怀孕结果。美国流行病学杂志，146, 1025-1035.

就职机构：北卡罗来纳大学公共卫生学院流行病学系；卡罗莱纳人口中心；加拿大疾控中心实验室中心，生育与儿童健康局

http://aje.oxfordjournals.org/content/146/12/1025.abstract

4) Claudia Bolognesi et al., Genotoxic Activity of Glyphosate and Its Technical Formulation Roundup, Journal of Agricultural and Food Chemistry 05/1997; 45(5). DOI: 10.1021/jf9606518
Claudia Bolognesi et al.，草甘膦及其配方制剂农达的细胞毒性活动性，农业与食物化学杂志，05/1997; 45(5).
http://www.researchgate.net/publication/231555170_Genotoxic_Activity_of_Glyphosate_and_Its_Technical_Formulation_Roundup

5) Lioi, M.B. et al., (1998). Cytogenetic damage and induction of pro-oxidant state in human lymphocytes exposed in vitro to glyphosate, vinclozolin, atrazine, and DPXE9636. Environmental and Molecular Mutagenesis, 32, 39-46.
Lioi, M.B. et al., (1998). 人类淋巴液试管中接触草甘膦、乙烯菌核利、秀去津与 DPXE9636 造成的细胞损伤与诱发强氧化状态
http://onlinelibrary.wiley.com/doi/10.1002/(SICI)1098-2280(1998)32:1%3C39::AID-EM5%3E3.0.CO;2-6/abstract

6) Peluso M, Munnia A, Bolognesi C, Parodi S. 32P-post labeling detection of DNA adducts in mice treated with the herbicide Roundup. Env Mol Mutagen. 1998;31:55-9. doi:10.1002/(SICI)1098-2280(1998)31:1<55::AID-EM8>3.0.CO;2-A.
Peluso M, Munnia A, Bolognesi C, Parodi S。配方制剂农达处理过的小鼠中的 DNA 加合物的 32P-后标记，环境分子诱变剂。1998;31:55-9.
http://www.ncbi.nlm.nih.gov/pubmed/9464316

7) Peggy J. Perkins at el., Toxicity of glyphosate and triclopyr using the frog embryo teratogenesis assay—Xenopu, Environmental Toxicology and Chemistry Volume 19, Issue 4, pages 940–945, April 2000

Peggy J. Perkins at el.，草甘膦与绿草定对非洲爪蟾蜍晶胚的毒性。环境性毒理学与化学，第 19 卷第 4 期 940-945，2000 年 4 月
就职机构：加拿大安大略 Guelph 大学环境生物学系
http://onlinelibrary.wiley.com/doi/10.1002/etc.5620190422/abstract

8) Walsh, L.P. et al., (2000). Roundup inhibits steroidogenesis by distrupting steroidogenic acute regulatory (StAR) protein expression. Environmental Health Perspectives, 108, 769-776.
Walsh, L.P. et al. (2000)。草甘膦除草剂农达通过干扰类固醇激素合成急性调节抑制(StAR)类固醇生成。环境健康前景，108, 769-776.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1638308/

9) Daruich, J. et al., (2001). Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. Environmental Research, 85, 226-231.
Daruich, J. et al, (2001)。草甘膦除草剂对怀孕鼠及其胎儿酶活性的影响。环境研究，85, 226-231.
就职机构：阿根廷国立圣路易斯大学生物学与医药系
http://www.ncbi.nlm.nih.gov/pubmed/11237511

10) T E Arbuckle et al., An exploratory analysis of the effect of pesticide exposure on the risk of spontaneous abortion in an Ontario farm population. Environ Health Perspect. 2001 August; 109(8): 851–857.
T E Arbuckle et al.，对安大略农场人口接触农药的自然流产风险影响的探索性分析
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1240415/

11) Marc J, Mulner-Lorillon O, Boulben S, Hureau D, Durand G, Bellé R. Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chem Res Toxicol. 2002;15(3):326-31.
http://www.ncbi.nlm.nih.gov/pubmed/11896679

12) Dallegrave, E. et al., (2003). The teratogenic potential of the herbicide glyphosate-Roundup in Wistar rats. Toxicology Letters, 142, 45-52.
Dallegrave, E. et al., (2003)。草甘膦除草剂对 Wistar 鼠的致畸潜力。毒理学通讯，142, 45-52.
就职机构：巴西国立 Rio Grande do Sul 大学，Ciências Básicas 救助学院，药学系
http://www.ncbi.nlm.nih.gov/pubmed/12765238
http://www.researchgate.net/publication/10745093_The_teratogenic_potential_of_the_herbicide_glyphosate-Roundup_(R)_in_Wistar_rats

13) Lajmanovich RC, Sandoval MT, Peltzer PM. Induction of mortality and malformation in Scinax nasicus tadpoles exposed to glyphosate formulations. Bull Env Contam Toxicol. 2003;70:612–618. doi:10.1007/s00128-003-0029-x.
Lajmanovich, R. C., Sandoval, M. T., Peltzer, P. M.，2003.暴露于草甘膦配方 Scinax nasicus 蝌蚪死亡与畸形的诱发。环境污染毒理学学报。70, 612–618.
http://www.researchgate.net/publication/10894330_Induction_of_mortality_and_malformation_in_Scinax_nasicus_tadpoles_exposed_to_glyphosate_formulations

14) Cox, C. (2004). Herbicide factsheet: glyphosate. Journal of Pesticide Reform, 24, 10-15.
Cox, C. (2004)。除草剂技术指标：草甘膦。农药改革杂志，24, 10-15.
http://www.mindfully.org/Pesticide/Roundup-Glyphosate-Factsheet-Cox.htm

15) Marc, J. et al., Glyphosate-based pesticides affects cell cycle regulation. Biology of the Cell (2004)., 96, 245-249.

Marc, J. et al.，草甘膦基除草剂影响细胞周期调制。细胞生物学(2004).,
96, 245-249.
就职机构：细胞周期发展生物站、国家科学研究所、法国皮尔与玛丽居里大学
http://www.ncbi.nlm.nih.gov/pubmed/15182708

16) Marc, J et al., (2004). Formulated glyphosate activities the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. Toxicological Sciences, 82, 436-442.
Marc, J et al., (2004)。草甘膦基配方除草剂激化细胞周期的 DNA 反应检查点导致阻止 G2/M 转变。毒理性科学，82, 436-442.
就职机构：细胞周期发展生物站、国家科学研究所、法国皮尔与玛丽居里大学
http://www.ncbi.nlm.nih.gov/pubmed/15375296

17) Benedettia, A.L. et al., (2004). The effects of sub-chronic exposure of Wistar rats to the herbicide Glyphosate-Biocarb. Toxicology Letters, 153, 227-232.
Benedettia, A.L. et al, (2004)。Wistar 鼠亚慢性接触草甘膦除草剂的影响。毒理学通讯，153, 227-232.
就职机构：巴西联邦 Santa Catarina Campus 大学，神经生物学与分子血液学实验室
http://www.ncbi.nlm.nih.gov/pubmed/15451553

18) John F Acquavella et al, Glyphosate biomonitoring for farmers and their families: results from the Farm Family Exposure Study. Environ Health Perspect. 2004 March; 112(3): 321–326.
John F Acquavella et al,对农民及其家人草甘膦的生物监察：农场家庭接触研究的结果。环境健康前景。2004 年 3 月；112(3): 321–326.
就职机构：流行病学部门，孟山都
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1241861/

19) Marc, J. et al., (2005). A glyphosate-based pesticide impinges on transcription. Toxicology and Applied Pharmacology, 203, 1-8.
Marc, J. et al., (2005)。草甘膦基除草剂对转录的侵害。毒理学与应用药理学，203, 1-8.
就职机构：细胞周期发展生物站、国家科学研究所、法国皮尔与玛丽居里大学
http://www.ncbi.nlm.nih.gov/pubmed/15694458

20) Lajmanovich, R.C.; Sandoval, M.T. & Peltzer, P.M. (2005). Induction of mortality and malformation in Scinax nasicus tadpoles exposed to glyphosate formulations. Bulletin of Environmental Contamination and Toxicology, 70, 612-618.
Lajmanovich, R.C. et al., 接触草甘膦除草剂在 Scinax nasicus 蝌蚪中诱发死亡率和畸形。环境污染和毒理学学报，70, 612-618.
http://www.researchgate.net/publication/10894330_Induction_of_mortality_and_malformation_in_Scinax_nasicus_tadpoles_exposed_to_glyphosate_formulations

21) Beuret CJ et al., Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses.
Reprod Toxicol. 2005 Mar-Apr;19(4):501-4.
Beuret CJ et al,草甘膦除草剂在怀孕鼠及其胎儿中对肝脂质过氧化的影响。繁殖毒理学杂志。2005 年 3 月-4 月；19(4):501-4.
就职机构：阿根廷国立圣路易斯大学生物学与药理学系
http://www.ncbi.nlm.nih.gov/pubmed/15749264

22) Richard S et al., Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect. 2005 Jun;113(6):716-20.

Richard S et al.，草甘膦与草甘膦除草剂浓度对人类胎盘细胞与芳香酶的差异性影响。环境性健康前景。2005 年 6 月；113(6):716-20.
就职机构：法国卡昂大学分子生物与生物化学实验室
http://www.ncbi.nlm.nih.gov/pubmed/15929894

23) Sparling DW et al., Toxicity of glyphosate as Glypro and LI700 to red-eared slider (trachemys scripta elegans) embryos and early hatchlings. Environ Toxicol Chem. 2006 Oct;25(10):2768-74.
Sparling DW et al.，草甘膦对红耳龟（耳龟）晶胚及其早期孵化的作为 Glypro 与 LI700
毒性，环境毒理学化学，2006 年 10 月；25(10):2768-74.
就职机构：南伊利诺伊大学动物学系生态中心合作性野生动物研究实验室
https://www.researchgate.net/publication/6773459_Toxicity_of_glyphosate_as_Glypro_and_LI700_to_red-eared_slider_(trachemys_scripta_elegans)_embryos_and_early_hatchlings

24) Oliveira AG et al., Effects of the herbicide Roundup on the epididymal region of drakes Anas platyrhynchos. Reprod Toxicol. 2007 Feb;23(2):182-91. Epub 2006 Nov 11.
Oliveira AG et al.，草甘膦除草剂农达对绿头鸭附睾部位的影响。繁殖毒理学。2007 年 2 月；23(2):182-91。网络发表：2006 年 11 月 11 日。
就职机构：巴西 Minas Gerais 联邦大学形态学系
http://www.ncbi.nlm.nih.gov/pubmed/17166697

25) César Paz-y-Miño et al., Evaluation of DNA damage in an Ecuadorian population exposed to glyphosate, Genetics and Molecular Biology, 01/2007; 30(2).
César Paz-y-Miño et al.，评价厄瓜多尔接触草甘膦人群的 DNA 损伤，遗传学与分子生物学，01/2007; 30(2).

https://www.researchgate.net/publication/237250392_Evaluation_of_DNA_damage_in_an_Ecuadorian_population_exposed_to_glyphosate

26) [Bellé R et al., Sea urchin embryo, DNA-damaged cell cycle checkpoint and the mechanisms initiating cancer development. J Soc Biol. 2007;201(3):317-27.]. [Article in French]
[Bellé R et al.海胆晶胚，DNA 损伤细胞周期检查点以及开始发展癌症的机制。生物学学会杂志； 2007;201(3):317-27.][原文系法文]
就职机构：国家科学研究中心
http://www.ncbi.nlm.nih.gov/pubmed/18157084

27) Dallegrave E et al., Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch Toxicol. 2007 Sep;81(9):665-73. Epub 2007 Jul 19.
Dallegrave E et al.商业草甘膦除草剂配方在 Wistar 鼠中的出生前与出生后毒性，毒理学档案。2007 年 9 月；81(9):665-73.网络出版：2007 年 7 月 19 日。
就职机构：巴西 Rio Grande do Sul 联邦大学，药理学系
http://www.ncbi.nlm.nih.gov/pubmed/17634926

28) McComb et al., (2008). Acute toxic hazard evaluations of glyphosate herbicide on terrestrial vertebrates of the Oregon Coast Range. Environmental Science and Pollution Research, 15, 266-272.
McComb et al., (2008)。草甘膦除草剂对俄勒冈海岸区域陆栖脊椎动物的畸形毒性危害评价。环境科学与污染研究，15, 266-272.
就职机构：俄勒冈州立大学森林科学系
http://www.ncbi.nlm.nih.gov/pubmed/18504846
http://cdn.intechopen.com/pdfs-wm/25624.pdf

29) Soso AB, Barcellos LJG, Ranzani-Paiva MJ, et al. Chronic exposure to sub-lethal concentration of a glyphosate-based herbicide alters

hormone profiles and affects reproduction of female Jundiá (Rhamdia quelen). Environ Toxicol Pharmacol. 2007;23:308–313.
http://www.sciencedirect.com/science/article/pii/S1382668906001578

30) Hokanson R, Fudge R, Chowdhary R, Busbee D. Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate. Hum Exp Toxicol. 2007;26:747-52. doi:10.1177/0960327107083453
http://www.ncbi.nlm.nih.gov/pubmed/17984146
http://het.sagepub.com/content/26/9/747.short

31) Mañas F, Peralta L, Raviolo J, et al. Genotoxicity of AMPA, the environmental metabolite of glyphosate, assessed by the Comet assay and cytogenetic tests. Ecotoxicol Env Saf. 2009;72:834-7. doi:10.1016/j.ecoenv.2008.09.019.
Mañas F, Peralta L, Raviolo J, et al. 草甘膦的环境性代谢物 AMPA 的细胞毒性，用彗星试验与细胞遗传学测试进行评估。生态毒理学环境性安全杂志。2009;72:834-7.
http://www.ncbi.nlm.nih.gov/pubmed/19013644

32) Nora Benachour and Gilles-Eric Séralini, Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells, *Chem. Res. Toxicol.*, 2009, *22* (1), pp 97–105
Nora Benachour and Gilles-Eric Séralini，草甘膦配方除草剂在人类脐带、胚芽与胎盘细胞诱发凋亡与坏疽，化学研究毒理学，2009, *22* (1), pp 97–105
摘要：http://pubs.acs.org/doi/abs/10.1021/tx800218n
全文：http://pubs.acs.org/doi/full/10.1021/tx800218n

33) Mariana, A. et al.,. (2009). The impact of simultaneous intoxication with agrochemicals on the antioxidant defense system in rat. Pesticide

Biochemistry and Physiology, 94, 93-99.

Mariana, A. et al.,. (2009)。农业化学品对老鼠抗氧化防御系统的同时中毒影响。农药生物化学与生理学，94, 93-99.

就职机构：阿根廷国立 La Plata 大学

http://www.sciencedirect.com/science/article/pii/S004835750900039X

34) Mañas F, Peralta L, Raviolo J, et al. Genotoxicity of glyphosate assessed by the Comet assay and cytogenic tests. Env Toxicol Pharmacol. 2009;28:37–41.

Mañas F, Peralta L, Raviolo J, et al.，草甘膦的细胞毒性，用彗星试验与细胞遗传学测试进行评估。环境毒理学药理学杂志。2009;28:37–41.

http://www.researchgate.net/publication/51516305_Genotoxicity_of_glyphosate_assessed_by_the_comet_assay_and_cytogenetic_tests

35) Prasad S et al., Clastogenic effects of glyphosate in bone marrow cells of swiss albino mice.

J Toxicol. 2009;2009:308985. Epub 2008 Dec 15.

Prasad S et al.，草甘膦在瑞士 albino 鼠骨髓细胞中的致染色体断裂影响。毒理学杂志。2009;2009:308985. Epub 2008-12-15.

就职机构：印度毒理学研究所，蛋白质组学实验室

http://www.ncbi.nlm.nih.gov/pubmed/20107585

36) Robin Mesnage et al., Two cases of birth defects overlapping the Stratton-Parker syndrome after multiple pesticide exposure, Letter, *Occup Environ Med* Published Online First 30 November 2009

Robin Mesnage et al.，多重农药接触后重叠患斯特拉顿帕克综合征的两例出生缺陷，通信，职业环境医学，网络首次发表：2009 年 11 月

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就职机构：法国卡昂大学

http://oem.bmj.com/content/early/2009/11/30/oem.2009.052969.abstract

37) Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Séralini GE. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 2009;262:184-91.
doi:10.1016/j.tox.2009.06.006.
Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Séralini GE。草甘膦除草剂在人类细胞系中致细胞毒性而且是内分泌干扰剂毒理学。2009;262:184-91.
http://www.ncbi.nlm.nih.gov/pubmed/19539684

38) Romano RM et al., Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Arch Toxicol. 2010 Apr;84(4):309-17. Epub 2009 Dec 12.
Romano RM et al.，青春期前接触商业草甘膦除草剂改变睾丸激素水平与睾丸形态。毒理学档案。2010 年 4 月；84(4):309-17. 网络发表 2009 年 12 月 12 日。
就职机构：巴西 Sao Paulo 大学兽医学院，动物生殖与荷尔蒙实验室剂量系
http://www.ncbi.nlm.nih.gov/pubmed/20012598

39) Jayawardene, U.A et al., (2010). Toxicity of agrochemicals to common hourglass tree frog (Polypedates crugiger) in acute and chronic exposure. International Journal of Agriculture and Biology, 12, 641-648.
Jayawardene, U.A et al., (2010)。农业化学品急性与慢性接触对普遍的沙漏树蛙（Polypedates crugiger）的毒性。国际农业与生物学杂志，12, 641-648.
http://www.fspublishers.org/ijab/past-issues/IJABVOL_12_NO_5/1.pdf

40) Paganelli, A.et al., (2010). Glyphosate- based herbicides produce teratogenic effects on vertebrates by impairing retionic acid signaling.

Chemical Research in Toxicology, 23, 1586-1595.
Paganelli, A.et al., (2010)。草甘膦基除草剂通过损伤损害视黄酸信号对脊椎动物造成畸形影响。毒理学化学研究，23, 1586-1595.
就职机构：阿根廷布宜诺斯艾利斯大学医学院胚胎分子学实验室
http://pubs.acs.org/doi/abs/10.1021/tx1001749

41) Rick A. Relyea. New effects of Roundup on amphibians: Predators reduce herbicide mortality; herbicides induce antipredator morphology. *Ecological Applications*, 2012; 22 (2): 634
DOI: 10.1890/11-0189.1
Rick A. Relyea。草甘膦除草剂对于脊椎动物新的作用：食肉动物减少除草剂死亡率；除草剂诱发抗食肉动物形态。生态应用，2012; 22 (2): 634
http://www.esajournals.org/doi/abs/10.1890/11-0189.1
中译文：美国科学家 2012 年论文：草甘膦除草剂可以诱发脊椎动物形态改变
http://blog.sina.com.cn/s/blog_4bb17e9d0102dzbm.html

42) Fabio Leonardo Meza-Joya et al., Toxic, cytotoxic, and genotoxic effects of a glyphosate formulation (Roundup®SL–Cosmoflux®411F) in the direct-developing frog *Eleutherodactylus johnstonei*, Environmental and Molecular Mutagenesis, Volume 54, Issue 5, pages 362–373,June 2013
Fabio Leonardo Meza-Joya et al.，草甘膦除草剂配方（Roundup®SL–Cosmoflux®411F）在直接发育的青蛙（*Eleutherodactylus johnstonei*）的毒性、细胞毒性以及基因毒性，环境与分子突变形成，第 54 卷第 5 期，362-373 页，2013 年 6 月
http://onlinelibrary.wiley.com/doi/10.1002/em.21775/abstract

43) Koller VJ, Furhacker M, Nersesyan A, Misik M, Eisenbauer M, Knasmueller S. Cytotoxic and DNA-damaging properties of glyphosate

and Roundup in human-derived buccal epithelial cells. Arch Toxicol. 2012;86:805–813. doi:10.1007/s00204-012-0804-8.
Koller VJ, Furhacker M, Nersesyan A, Misik M, Eisenbauer M, Knasmueller S. 草甘膦与农达在人类口腔上皮细胞中造成细胞毒性与 DNA 损伤的性质。毒理学档案。2012;86:805–813.
http://link.springer.com/article/10.1007%2Fs00204-012-0804-8

44) Vandenberg LN, Colborn T, Hayes TB, et al. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev. 2012;33(3):378-455. doi:10.1210/er.2011-1050.
Vandenberg LN, Colborn T, Hayes TB, et al. 激素与内分泌干扰化学品：低剂量效应与**非单调性剂量响应。内分泌审视。**
2012;33(3):378-455.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3365860/

45) Benedetti D, Nunes E, Sarmento M, et al. Genetic damage in soybean workers exposed to pesticides: evaluation with the comet and buccal micronucleus cytome assays. Mutat Res. 2013;752:28-33. doi:10.1016/j.mrgentox.2013.01.001.
Benedetti D, Nunes E, Sarmento M, et al.暴露于除草剂的大豆种植工人中的遗传毒性：用彗星试验与颊微核细胞组学试验进行评价。突变研究。2013;752:28-33.
http://www.sciencedirect.com/science/article/pii/S138357181300003X

46) Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J. Glyphosate induces human breast cancer cells growth via estrogen receptors. Food Chem Toxicol. 2013. doi:10.1016/j.fct.2013.05.057.

Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J.**草甘膦通过雌激素受体蛋白诱发人类乳房癌细胞生长。食物化学毒理学。2013 年 6 月 9 日发布**

http://www.ncbi.nlm.nih.gov/pubmed/23756170

附录 4：国外学者研究发现草甘膦是内分泌干扰剂 17 项科学试验证据！

Attachment 4: Seventeen studies show evidence that glyphosate is an Endocrine Disrupting Chemical **(EDC**).

国外学者研究发现草甘膦是内分泌干扰剂 17 项科学试验证据。农业部与中国疾控中心沿用传统毒理学已经过时的"剂量决定毒性"错误观念，故意无视草甘膦等内分泌干扰剂这样的化学品在极低微量危害人类的一系列内分泌激素，对健康造成终生多方面系统性损害！

The Ministry of Agriculture and the China Disease Prevention & Control Center (China CDC) continue to use the out of date "dosage decides toxicity" concept of traditional toxicology, purposely ignoring that chemicals like glyphosate and other EDCs, at very low level cause harm to a series of hormone systems of humans, causing life-long systematic harm in many aspects!

要点概述

Summary of Main Points

中国学者任晋、蒋可 2001 年《内分泌干扰剂的研究进展》摘要：内分泌干扰剂（EDC）正在成为生态环境研究的前沿课题，并受到各国政府的密切关注。本文综述了内分泌干扰剂的危害、作用机理、化合物类型及研究进展，特别强调了化合物低剂量长期暴露潜在危害的新概念，详述了传统的环境毒理学和环境分析化学所遇到的挑战及生物分析、化学仪器分析和生物传感器技术在内分泌干扰剂筛选过程中的重要战略地位。

"Advances of Endocrine Disrupting Chemicals Research" by Chinese scholar Ren Jin, Jiang Ke, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences. Summary: EDC is becoming a front-edge topic of ecological environment studies, and receiving close attention by governments of each nation. This paper summarizes advances in the studies of the harms caused by EDCs, their mechanism, kinds of compounds, and especially emphasizes on the new concept of potential harm caused by low concentrations but long-term exposure to such chemical compounds, discussed in detail the challenge traditional environment toxicology and environment analysis chemistry has faced, the important strategic position of biological analysis, chemical instrument analysis and biosensors technology in the screening process of EDCs.

"Endocrine Disruptor Screening Program (EDSP)" issued on April15，2009 by EPA points out:

美国环境保护署（**EPA**）**2009** 年 **4** 月 **15** 日《内分泌干扰剂筛选程序（**EDSP**）》指出：

In the 1990's, some scientists proposed that certain chemicals might be disrupting the endocrine systems of humans and wildlife. A variety of chemicals have been found to disrupt the endocrine systems of animals in laboratory studies, and compelling evidence shows that endocrine systems of certain fish and wildlife have been affected by chemical contaminants, resulting in developmental and reproductive problems.

90 年代，某些科学家提议某些化学品可能对人类与野生动物内分泌系统造成干扰。在实验室研究总发现一系列化学品对动物内分泌系统造成干扰，令人信服的证据表明某些鱼类与野生动物的内分泌系统遭到化学污染的影响，导致发育与繁殖性问题。

Based on this and other evidence, Congress passed the Food Quality Protection Act and the Safe Drinking Water Act (SDWA) Amendments in 1996 requiring that EPA screen pesticide chemicals for their potential to produce effects similar to those produced by the female hormones (estrogen) in humans and giving EPA the authority to screen certain other chemicals and to include other endocrine effects. Based on recommendations from an Advisory Committee, EPA has expanded the EDSP to include male hormones (androgens) and the thyroid system, and to include effects on fish and wildlife.

基于此以及其他的证据，国会 **1996** 年通过了《食品质量保护法》与《安全饮水法》修订案，要求环境保护署对于农药化学品产生雌性激素（雌激素）

在人类中造成同样影响的潜在可能进行筛选，并且授权环境保护署对某些其他的化学品，并包括其他内分泌影响，进行筛选。基于一个咨询委员会的推荐意见，环境保护署扩展了 **EDSP**（内分泌干扰剂筛选程序），将男性激素（雄激素）以及甲状腺系统包括进来，同时包括对鱼类与野生动物的影响。

"Environmental causes of cancer: endocrine disruptors as carcinogens" published in 2010 by *Nature Reviews Endocrinology* emphasizes:

《自然杂志》**2010** 年《致癌环境性原因：致癌的内分泌干扰剂，自然内分泌腺审查》强调：

Environmental endocrine disrupting chemicals (EDCs), including pesticides and industrial chemicals, have been and are released into the environment producing deleterious effects on wildlife and humans. The effects observed in animal models after exposure during organogenesis correlate positively with an increased incidence of malformations of the male genital tract and of neoplasms and with the decreased sperm quality observed in European and US populations. Exposure to EDCs generates additional effects, such as alterations in male and female reproduction and changes in neuroendocrinology, behavior, metabolism and obesity, prostate cancer and thyroid and cardiovascular endocrinology. This Review highlights the carcinogenic properties of EDCs, with a special focus on bisphenol A. However, humans and wildlife are exposed to a mixture of EDCs that act contextually. To explain this mindboggling complexity will require the design of novel experimental approaches that integrate the effects of different doses of structurally different chemicals that act at different ages on different target tissues.

环境性内分泌干扰化学品（**EDC**），包括农药与工业化学品，释放到环境中对野生动物与人类造成有害影响。动物模型中观察到的接触内分泌干扰化学品后器官形成中发现的影响，与欧洲与美国人口中观察到的男性生殖系统及其赘生物与精子治疗降低的状况正向关联。接触环境性内分泌干扰化学品还产生其他的影响，例如男性与女性繁殖中的改变、神经内分泌、行为、新陈代谢与肥胖症、前列腺癌与甲状腺与心血管内分泌异常。

该审查汇集环境性内分泌干扰化学品的致癌性质，并特别关注双酚的作用。然而，人类与野生动物接触多种相互作用的环境性内分泌干扰化学品的混合体。为了解释这种极费脑筋的复杂状况，要求设计新的实验方法，综合考虑不同结构不同剂量不同化学品的作用，它们在不同的年龄段对不同靶标造成不同的影响。

Vandenberg LN et al.（2012）reveals: For decades, studies of endocrine-disrupting chemicals (EDCs) have challenged traditional concepts in toxicology, in particular the dogma of “the dose makes the poison,” because EDCs can have effects at low doses that are not predicted by effects at higher doses. Here, we review two major concepts in EDC studies: low dose and nonmonotonicity. ... We conclude that when nonmonotonic dose-response curves occur, the effects of low doses cannot be predicted by the effects observed at high doses. Thus, fundamental changes in chemical testing and safety determination are needed to protect human health.

Vandenberg LN et al.（2012）揭示：传统毒理学一直坚持的法则。近几十年，内分泌干扰化学品（**EDC**）的研究对毒理学传统概念“剂量致毒性”的法则提出了挑战，因为内分泌干扰化学品（**EDC**）高剂量的影响无法预测低剂量的影响。我们审查了内分泌干扰化学品（**EDC**）研究中的两项主要概念：低剂量与非单调性（**nonmonotonicity**）。**...** 我们结论，非单调性剂量响应曲线发生时，低剂量时的效应无法由高剂量观察到的效应进行预测。因此，对毒理学中化学测试与毒性确定需要做实质性改变来保护人类安全。

The EPA on April 14, 2009 announced the preliminary list for the Endocrine Disruptor Screening Program, which included glyphosate.

环境保护署 **2009** 年 **4** 月 **14** 日宣布了对内分泌系统潜在影响进行筛选的化学品初步清单，其中包括草甘膦。

国外学者研究发现草甘膦是内分泌干扰剂 **17** 项科学试验证据！

Oversea 17 studies find evidence that glyphosate is an EDC!

1）Yousef MI et al. (1995): Pesticide treatment resulted in a decline in body weight, libido, ejaculate volume, sperm concentration, semen initial fructose and semen osmolality. This was accompanied with increases in the abnormal and dead sperm and semen methylene blue reduction time. The hazardous effect of these pesticides on semen quality continued during the recovery period, and was dose-dependent. These effects on sperm quality may be due to the direct cytotoxic effects of these pesticides on spermatogenesis and/or indirectly via hypothalami-pituitary-testis axis which control the reproductive efficiency.

1）Yousef MI et al.（1995）：草甘膦造成实验兔体重、性欲、射精量、精子浓度等指标下降，危害精子质量的作用终止处理后继续发展，而且剂量依赖。机理可能是草甘膦对精子声称的直接细胞毒性，和/或间接通过控制繁殖效率的视丘下部垂体睾丸轴造成这些损害。

2)Walsh, L.P. et al. (2000): The pesticide Roundup inhibited dibutyryl [(Bu)(2)]cAMP-stimulated progesterone production in MA-10 cells without causing cellular toxicity. Roundup inhibited steroidogenesis by disrupting StAR protein expression, further demonstrating the susceptibility of StAR to environmental pollutants.

2）Walsh, L.P. et al.**（2000）：在小鼠肿瘤细胞中发现草甘膦除草剂农达抑制参与性激素蛋白质生物合成活动。这把胆固醇 -- 妊娠烯醇酮 -- 孕激素转换途径的运行降低到最低水平。**

3) Marc J et al. (2002): In summary, Roundup affects cell cycle regulation by delaying activation of the CDK1/cyclin B complex, by synergic effect of glyphosate and formulation products. Considering the universality among species of the CDK1/cyclin B regulator, our results question the safety of glyphosate and Roundup on human health.

3）Marc J et al.**（2002）：简要讲，农达通过草甘膦及其配方制剂的协同效应延迟 CDK1/细胞周期蛋白 B 的活动性影响细胞的生长周期。考虑到不**

同物种中 CDK1/细胞周期蛋白 B 调制器的普遍性，我们质疑草甘膦与农达对人类健康的安全性。

4) Marc, J et al. (2004): At a concentration that efficiently impeded the cell cycle, formulated glyphosate inhibited the synthesis of DNA occurring in S phase of the cell cycle. The extent of the inhibition of DNA synthesis by formulated glyphosate was correlated with the effect on the cell cycle. We conclude that formulated glyphosate's effect on the cell cycle is exerted at the level of the DNA-response checkpoint of S phase. The resulting inhibition of CDK1/cyclin B Tyr 15 dephosphorylation leads to prevention of the G2/M transition and cell cycle progression.

4）Marc, J et al.（**2004）：草甘膦除草剂抑制首个细胞周期的 G2/M 阶段的 DNA 生物合成。**

5) Marc, J et al. (2004): Roundup Biovert induced cell cycle dysfunction. The threshold concentration for induction of cell cycle dysfunction was evaluated for each product and suggests high risk by inhalation for people in the vicinity of the pesticide handling sprayed at 500 to 4000 times higher dose than the cell-cycle adverse concentration.

5）Marc, J et al.（2004）**：农业喷洒剂量稀释 500 至 4000 倍的草甘膦除草剂导致发展癌症的细胞周期机能失调。**

6) Beuret CJ et al (2005): The present study has investigated the effects that 1% glyphosate oral exposure has on lipoperoxidation and antioxidant enzyme systems in the maternal serum and liver of pregnant rats and their term fetuses at 21 days of gestation. The results suggest that excessive lipid peroxidation induced with glyphosate ingestion leads to an overload of maternal and fetal antioxidant defense systems.

6）Beuret CJ et al（2005）：研究试验口服 1%浓度草甘膦在 21 天孕期中对怀孕鼠的血清与肝及其胎儿的脂质过氧化与抗氧化酶系统的影响。结果

发现，摄入草甘膦诱发过量脂质过氧化，导致对怀孕鼠及其胎儿抗氧化防御系统过量。

7) **Richard S et al. (2005):** We tested the effects of glyphosate and Roundup at lower nontoxic concentrations on aromatase, the enzyme responsible for estrogen synthesis. The glyphosate-based herbicide disrupts aromatase activity and mRNA levels and interacts with the active site of the purified enzyme, but the effects of glyphosate are facilitated by the Roundup formulation in microsomes or in cell culture. We conclude that endocrine and toxic effects of Roundup, not just glyphosate, can be observed in mammals. We suggest that the presence of Roundup adjuvants enhances glyphosate bioavailability and/or bioaccumulation.

7）Richard S et al.（2005）在哺乳动物中可以观察到草甘膦除草剂农达的内分泌干扰与毒性影响，不仅是草甘膦的内分泌干扰与毒性影响。我们认为农达的辅佐剂强化了的草甘膦的生物可获得性和/或生物积蓄。

8) Oliveira AG et al. (2007): The exposure to the herbicide resulted in alterations in the structure of the testis and epididymal region as well as in the serum levels of testosterone and estradiol, with changes in the expression of androgen receptors restricted to the testis. The harmful effects were more conspicuous in the proximal efferent ductules and epididymal ducts, suggesting higher sensitivity of these segments among the male genital organs. The effects were mostly dose dependent, indicating that this herbicide may cause disorder in the morphophysiology of the male genital system of animals.

8）Oliveira AG et al.（2007）：接触草甘膦除草剂造成对睾丸与附睾区域构造的改变，还改不了血清中睾丸激素与雌二醇的水平，改变了睾丸雄性激素受体的表达。更明显的近端传对微胆管和附睾导管造成有害影响，表明雄生殖器中这些部位对草甘膦更为敏感。造成的影响具有剂量依赖性，表明草甘膦除草剂可能在动物雄生殖系统中造成形态生理学失调。

9) Dallegrave E et al. (2007): The results showed that glyphosate-Roundup did not induce maternal toxicity but induced adverse reproductive effects on male offspring rats: a decrease in sperm number per epididymis tail and in daily sperm production during adulthood, an increase in the percentage of abnormal sperms and a dose-related decrease in the serum testosterone level at puberty, and signs of individual spermatid degeneration during both periods. There was only a vaginal canal-opening delay in the exposed female offspring. These findings suggest that in utero and lactational exposure to glyphosate-Roundup may induce significant adverse effects on the reproductive system of male Wistar rats at puberty and during adulthood.

9）Dallegrave E et al.（2007）：研究结果表明，草甘膦除草剂农达没有诱发雌鼠毒性，但是对雄性后代造成了有害繁殖性影响：鼠仔成年后每个附睾精子数量与每天精子产生量减少、增加异常精子百分比，进入青春期后血清睾丸激素水平产生与草甘膦接触量相关减少，同时在两个阶段出现精子退化迹象。后代雌鼠仔长大后仅发生阴道开通延迟。这些发现表明，子宫内与哺乳期接触草甘膦除草剂农达可能对雄鼠仔进入青春期与成年期阶段的生殖系统诱发显著有害影响。

10) Nora Benachour et al. (2009): We have evaluated the toxicity of four glyphosate (G)-based herbicides in Roundup (R) formulations, from 105 times dilutions, on three different human cell types. This dilution level is far below agricultural recommendations and corresponds to low levels of residues in food or feed. The formulations have been compared to G alone and with its main metabolite AMPA or with one known adjuvant of R formulations, POEA. HUVEC primary neonate umbilical cord vein cells have been tested with 293 embryonic kidney and JEG3 placental cell lines. All R formulations cause total cell death within 24 h, through an inhibition of the mitochondrial succinate dehydrogenase activity, and necrosis, by release of cytosolic adenylate kinase measuring membrane damage. They also induce apoptosis via activation of enzymatic caspases 3/7 activity. This is confirmed by characteristic DNA fragmentation, nuclear shrinkage (pyknosis), and nuclear fragmentation (karyorrhexis), which is demonstrated by DAPI in apoptotic round cells. G provokes only apoptosis, and HUVEC are 100 times more sensitive overall at this level. The deleterious effects are not proportional to G concentrations but rather depend on the nature of the adjuvants. AMPA and POEA separately and synergistically damage cell membranes like R but at different concentrations. Their mixtures are generally even more harmful with G. In conclusion, the R adjuvants like POEA change human cell permeability and amplify toxicity induced already by G, through apoptosis and necrosis. The real threshold of G toxicity must take into account the presence of adjuvants but also G metabolism and time-amplified effects or bioaccumulation. This should be discussed when analyzing the in vivo toxic actions of R. This work clearly confirms that the adjuvants in Roundup formulations are not inert. Moreover, the proprietary mixtures available on the market could cause cell damage and even death around residual levels to be expected, especially in food and feed derived from R formulation-treated crops.

10）Nora Benachour et al.（**2009**）**:**对草甘膦及其主要代谢物 **AMPA**，以及草甘膦添加除草剂配方中主要辅佐剂 **POEA**（表面活性剂，稀释 **10** 万倍，对三种不同的人类细胞的毒性。这样的稀释水平，远远低于农业应用推荐的水平，并对应于食品或者饲料中草甘膦残留的低水平。三种人类细胞分别为新生儿脐带静脉的细胞、**293** 胚肾细胞与 **JEG3** 胎盘细胞系。所有草甘膦的配方在 **24** 小时内造成所有细胞死亡，通过抑制线粒体琥珀酸脱氢酶活性，以及通过释放胞质膜伤害腺苷酸激酶测量膜损伤导致坏疽。通过激化酶的半胱天冬酶 **3/7** 活性诱发细胞凋亡。单独草甘膦激起仅细胞凋亡，而人脐静脉内皮细胞（**HUVEC**）在这个水平上 **100** 倍更敏感。有害效应与草甘膦浓度不成比例，更加取决于除草剂辅佐剂的性质。草甘膦代谢物 **AMPA** 与 **POEA**（草甘膦除草剂配方中的表面活性剂），分别单独作用或者合在一起协作用时，像草甘膦一样，损伤细胞膜，但是各自在不同的浓度发挥作用。它们与草甘膦一起的混合物的危害通常更强。结论，像 **POEA**

这样的辅佐剂，改变人类细胞的渗透性，以凋亡与坏疽方式强化诱发草甘膦诱发的毒性。草甘膦真正的阈值，必须考虑存在的辅佐剂，还必须考虑草甘膦的代谢、时间放大效应或生物蓄积作用。市场上销售的草甘膦除草剂，即便在抗草甘膦作物加工的食品与饲料残留水平，能够造成细胞损伤以至死亡。

11) Gasnier C et al. (2009): Glyphosate-based herbicides are the most widely used across the world; they are commercialized in different formulations. Their residues are frequent pollutants in the environment. In addition, these herbicides are spread on most eaten transgenic plants, modified to tolerate high levels of these compounds in their cells. Up to 400 ppm of their residues are accepted in some feed. We exposed human liver HepG2 cells, a well-known model to study xenobiotic toxicity, to four different formulations and to glyphosate, which is usually tested alone in chronic in vivo regulatory studies. We measured cytotoxicity with three assays (Alamar Blue, MTT, ToxiLight), plus genotoxicity (comet assay), anti-estrogenic (on ERalpha, ERbeta) and anti-androgenic effects (on AR) using gene reporter tests. We also checked androgen to estrogen conversion by aromatase activity and mRNA. All parameters were disrupted at sub-agricultural doses with all formulations within 24h. These effects were more dependent on the formulation than on the glyphosate concentration. First, we observed a human cell endocrine disruption from 0.5 ppm on the androgen receptor in MDA-MB453-kb2 cells for the most active formulation (R400), then from 2 ppm the transcriptional activities on both estrogen receptors were also inhibited on HepG2. Aromatase transcription and activity were disrupted from 10 ppm. Cytotoxic effects started at 10 ppm with Alamar Blue assay (the most sensitive), and DNA damages at 5 ppm. A real cell impact of glyphosate-based herbicides residues in food, feed or in the environment has thus to be considered, and their classifications as carcinogens/mutagens/reprotoxics is discussed.

11) Gasnier C et al.（**2009**）**:**草甘膦为基础草甘膦在世界上最广泛使用。

它们的残留成为环境中经常有的污染物。此外，这些除草剂还喷洒到最大量食用的转基因作物，这样的作物使其容忍细胞中高水平的这些成分。某些饲料中允许它们高达 **400 ppm** 残留。人类肝脏 **HepG2** 细胞是研究异型生物质毒性的知名模型，我们让人类肝脏 **HepG2** 细胞接触草甘膦及其四种不同配方除草剂制剂。通常仅在慢性活体内对单独草甘膦成分进行试验。我们用三种试验方法（**Alamar Blue, MTT, ToxiLight**），以及基因毒性（彗星试验）、抗雌激素（对 **ERalpha, ERbeta**）与抗雄激素效果（对 **AR**）做基因检测试验。我们还用芳香化酶活性与 **mRNA** 检测雄激素雌激素转换。所有指标在 **24** 小时内都受到亚农业用剂量用的草甘膦及其四种配方除草剂制剂所有成分的干扰。其效果更依赖于草甘膦除草剂的配方而非草甘膦的剂量。首先观察到的人类细胞内分泌干扰是最为活性配方制剂（**R400**）从 **0.5 ppm** 剂量在 **MDA-MB453-kb2** 细胞中对雄激素受体的作用，然后从 **2 ppm** 剂量起，**HepG2** 细胞的两个雌激素受体的转录活动性遭到抑制。从 **10 ppm** 剂量起，芳香化酶转录和活动收到干扰。在 **Alamar Blue** 试验（最敏感的）中，从 **10 ppm** 剂量其发生细胞毒性作用，但从 **5 ppm** 起发生 **DNA** 损伤。因此必须考虑食物、饲料或者环境中草甘膦除草剂对真实细胞的影响，对草甘膦分类为致癌物/致突变/致生殖毒性进行了讨论。

12) Romano RM et al. (2010): These results suggest that commercial formulation of glyphosate is a potent endocrine disruptor in vivo, causing disturbances in the reproductive development of rats when the exposure was performed during the puberty period.

12）Romano RM et al.（2010）:研究结果表明商业配方的草甘膦除草剂在体内是一种威力强大的内分泌干扰剂，青春期接触时对老鼠的生育系统发育造成干扰。

13) Jayawardene, U.A et al.(2010): Glyphosate recorded the highest percentage of malformation (69%) compared to other pesticides in 1.00 ppm concentration. Malformations observed were mainly in the spine, such as hunched back (kyphosis) and curvature (scoliosis), while edema and skin ulcers were also observed

13）Jayawardene, U.A et al.（**2010**）**:**用 **1ppm** 浓度，草甘膦对沙漏树蛙蝌蚪处理后，造成几种农药中最高的 **69%**畸形率。观察到的畸形主要为脊柱畸形，如驼背（驼背）和曲率（脊柱侧弯），也观察到而水肿和皮肤溃疡。

39) (2010):

14) Paganelli, A.et al. (2010): Xenopus laevis embryos were incubated with 1/5000 dilutions of a commercial GBH. The treated embryos were highly abnormal with marked alterations in cephalic and neural crest development and shortening of the anterior–posterior (A-P) axis. Alterations on neural crest markers were later correlated with deformities in the cranial cartilages at tadpole stages. Embryos injected with pure glyphosate showed very similar phenotypes. Moreover, GBH produced similar effects in chicken embryos, showing a gradual loss of rhombomere domains, reduction of the optic vesicles, and microcephaly. This suggests that glyphosate itself was responsible for the phenotypes observed, rather than a surfactant or other component of the commercial formulation. A reporter gene assay revealed that GBH treatment increased endogenous retinoic acid (RA) activity in Xenopus embryos and cotreatment with a RA antagonist rescued the teratogenic effects of the GBH. Therefore, we conclude that the phenotypes produced by GBH are mainly a consequence of the increase of endogenous retinoid activity.

14）Paganelli, A.et al.（**2010**）**:**非洲爪蟾蜍晶胚与稀释 **5000** 倍草甘膦除草剂一起孵化。经处理的晶胚高度异常，头盖与神经嵴明显改变，前后轴缩短。改变后神经嵴标记与颅软骨蝌蚪阶段畸形一致。注射单独草甘膦显示非常类似的畸形。草甘膦除草剂在鸡晶胚中显示类似的影响，显示一个逐渐失去了菱域。减少视觉囊泡和小头畸形。这表明草甘膦自己对观察到的畸形负责，而不是草甘膦除草剂配方中的表面活性剂或其他组分。一个报告基因分析，揭示草甘膦除草剂处理增加了非洲爪蟾蜍晶胚中的视黄酸活性，而且与 **RA** 拮抗剂的协作处理保持了草甘膦除草剂的致畸效应。因此结论草甘膦除草剂产生的显型主要是内源性类活动增加的结果。

15) Koller VJ (2012): Comparisons with results of earlier studies with lymphocytes and cells from internal organs indicate that epithelial cells are more susceptible to the cytotoxic and DNA-damaging properties of the herbicide and its formulation. Since we found genotoxic effects after short exposure to concentrations that correspond to a 450-fold dilution of spraying used in agriculture, our findings indicate that inhalation may cause DNA damage in exposed individuals.

15）Koller VJ（**2012**）**:与早期的草甘膦对内部器官淋巴与细胞的研究进行比较表明，上皮细胞更容易受到细胞毒性，而且 DNA 损伤的性质与除草剂及其配方相关。由于我们发现农业中喷洒剂量稀释 450 倍的短期暴露造成基因毒性的影响，我们的发现表明，喷洒草甘膦除草剂可能造成吸入人类 DNA 损伤。**

16) R. Mesnagea (2013): Among them, POE-15 clearly appears to be the most toxic principle against human cells, even if others are not excluded. It begins to be active with negative dose-dependent effects on cellular respiration and membrane integrity between 1 and 3 ppm, at environmental/occupational doses. We demonstrate in addition that POE-15 induces necrosis when its first micellization process occurs, by contrast to glyphosate which is known to promote endocrine disrupting effects after entering cells.

16）R. Mesnagea（**2013**）**：在它们之中，（农达配方中的表面活性剂）POE-15 清楚显示是对人类细胞最为毒性的成分，即便不排除其他成分。他始于环境性/职业接触剂量 1 - 3 ppm 之间对细胞呼吸与细胞膜整体性负面的剂量依赖作用。我们演示了 POE-15 还在它的首次胶束化作用发生时诱发坏疽，这与草甘膦进入细胞后促进内分泌干扰作用有所不同。**

17) Thongprakaisang S (2013): Glyphosate is an active ingredient of the most widely used herbicide and it is believed to be less toxic than other pesticides. However, several recent studies showed its potential adverse health effects to humans as it may be an endocrine disruptor. This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10^{-12} to 10^{-6} M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity. Glyphosate-based herbicides are widely used for soybean cultivation, and our results also found that there was an additive estrogenic effect between glyphosate and genistein, a phytoestrogen in soybeans. However, these additive effects of glyphosate contamination in soybeans need further animal study.

17）Thongprakaisang S（**2013**）：草甘膦是最为广泛使用并被人们相信比其他农药毒性较低的除草剂的活性成分。然而，最近几项研究显示它对人类潜在危害健康，而且可能是一种内分泌干扰剂。该项研究集中于纯草甘膦对雌激素受体蛋白（**estrogen receptors**）促进的转录活动性及其表达。草甘膦，在 **10^{-12}** 至 **10^{-6} M**（万亿分之一至百万分之一质量）范围，在雌激素撤走状态下仅对人类激素-依赖乳房癌细胞 **T47D** 细胞发挥增殖作用，而对激素-独立乳房癌细胞 **MDA-MB231** 细胞系没有这种作用。诱发 **ERE**（雌激素反应元素）转录活动性达到 **T47D-KBluc** 细胞中对照组的 **5-13** 倍的产生增殖作用剂量的草甘膦，受到一种雌激素对抗药 **ICI 182780** 的抑制，表明草甘膦的雌激素活动性通过雌激素受体蛋白（**estrogen receptors**）发挥作用。此外，草甘膦既改变雌激素受体蛋白（**estrogen receptors**）**α**，有改变其 **β** 表达。这些结果显示，在低的与环境性相关浓度下，草甘膦具有雌激素性活动。草甘膦为基础的除草剂广泛用于（转基因）大豆种植，而我们的结果发现，存在着草甘膦与大豆中的一种植物雌激素染料木黄酮

（genistein）之间的额外雌激素作用。然而，草甘膦对于（转基因）大豆的这种额外的作用需要进行进一步的动物试验。

任晋、蒋可，内分泌干扰剂的研究进展，化学进展，2001, 13(2)

作者就职机构：中国科学院生态环境研究中心

http://d.wanfangdata.com.cn/Periodical_hxjz200102009.aspx

摘要：内分泌干扰剂（EDC）正在成为生态环境研究的前沿课题，并受到各国政府的密切关注。本文综述了内分泌干扰剂的危害、作用机理、化合物类型及研究进展，特别强调了化合物低剂量长期暴露潜在危害的新概念，详述了传统的环境毒理学和环境分析化学所遇到的挑战及生物分析、化学仪器分析和生物传感器技术在内分泌干扰剂筛选过程中的重要战略地位。

美国环境保护署：内分泌干扰剂筛选程序（EDSP）– 2009 年 4 月 15 日

U.S. Environmental Protection Agency: Endocrine Disruptor Screening Program (EDSP) -- April 15, 2009

http://www.epa.gov/endo/

90 年代，某些科学家提议某些化学品可能对人类与野生动物内分泌系统造成干扰。在实验室研究总发现一系列化学品对动物内分泌系统造成干扰，令人信服的证据表明某些鱼类与野生动物的内分泌系统遭到化学污染的影响，导致发育与繁殖性问题。基于此以及其他的证据，国会 1996 年通过了《食品质量保护法》与《安全饮水法》修订案，要求环境保护署对于农药化学品产生雌性激素（雌激素）在人类中造成同样影响的潜在可能进行筛选，并且授权环境保护署对某些其他的化学品，并包括其他内分泌影响，进行筛选。基于一个咨询委员会的推荐意见，环境保护署扩展了 EDSP（内分泌干扰剂筛选程序），将男性激素（雄激素）以及甲状腺系统包括进来，同时包括对鱼类与野生动物的影响。

环境保护署 2009 年 4 月 14 日宣布了对内分泌系统潜在影响进行筛选的化学品初步清单（或第一层次试验），并且于 2009 年 10 月 29 日发布了第一批试验指令。试验指令是要求提供数据。现在，筛选正在进行，环境保护署正在对试验指令回应进行审查，并且正在对有关状态或试验指令回

应和/或有关试验要求的任何决定做出允许了解的安排。

《自然杂志》致癌环境性原因：致癌的内分泌干扰剂，自然内分泌腺审查，2010

Ana M. Soto & **Carlos Sonnenschein,** Environmental causes of cancer: endocrine disruptors as carcinogens, *Nature Reviews Endocrinology* **6**, 363–370 (1 July 2010)

http://www.nature.com/nrendo/journal/v6/n7/authors/nrendo.2010.87.html

环境性内分泌干扰化学品（EDC），包括农药与工业化学品，释放到环境中对野生动物与人类造成有害影响。动物模型中观察到的接触内分泌干扰化学品后器官形成中发现的影响，与欧洲与美国人口中观察到的男性生殖系统及其赘生物与精子治疗降低的状况正向关联。接触环境性内分泌干扰化学品还产生其他的影响，例如男性与女性繁殖中的改变、神经内分泌、行为、新陈代谢与肥胖症、前列腺癌与甲状腺与心血管内分泌异常。该审查汇集环境性内分泌干扰化学品的致癌性质，并特别关注双酚的作用。然而，人类与野生动物接触多种相互作用的环境性内分泌干扰化学品的混合体。为了解释这种极费脑筋的复杂状况，要求设计新的实验方法，综合考虑不同结构不同剂量不同化学品的作用，它们在不同的年龄段对不同靶标造成不同的影响。

（2012）：传统毒理学一直坚持的法则。近几十年，内分泌干扰化学品（EDC）的研究对毒理学传统概念“剂量致毒性”的法则提出了挑战，因为内分泌干扰化学品（EDC）高剂量的影响无法预测低剂量的影响。我们审查了内分泌干扰化学品（EDC）研究中的两项主要概念：低剂量与非单调性（nonmonotonicity）。... 我们结论，非单调性剂量响应曲线发生时，低剂量时的效应无法由高剂量观察到的效应进行预测。因此，对毒理学中化学测试与毒性确定需要做实质性改变来保护人类安全。

Vandenberg LN, Colborn T, Hayes TB, et al. Hormones and endocrine-disrupting chemicals: Low-dose effects and nonmonotonic dose responses. Endocr Rev. 2012;33(3):378-455. doi:10.1210/er.2011-1050.
Vandenberg LN, Colborn T, Hayes TB, et al. 激素与内分泌干扰化学品：低剂量效应与非单调性剂量响应。内分泌审视。2012;33(3):378-455.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3365860/

美国环境保护署：2009 年 4 月进行第一层次筛选化学品的最终清单：包括草甘膦！
U.S. EPA: April 2009 Final List of Chemicals for Initial Tier 1 Screening: Glyphosate
http://www.epa.gov/endo/pubs/prioritysetting/finallist.html

科学证据 1（1995）：草甘膦造成实验兔体重、性欲、射精量、精子浓度等指标下降，危害精子质量的作用终止处理后继续发展，而且剂量依赖。机理可能是草甘膦对精子声称的直接细胞毒性，和/或间接通过控制繁殖效率的视丘下部垂体睾丸轴造成这些损害。

1) Yousef MI et al., Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. J Environ Sci Health B. 1995 Jul;30(4):513-34.
Yousef MI et al.，呋喃丹与草甘膦对兔子精子特征的毒性影响，环境科学健康学报。1995 年 7 月，30(4):513-34.
就职机构：埃及亚历山大大学环境研究系
http://www.ncbi.nlm.nih.gov/pubmed/7797819

科学证据 2（2000 年）：在小鼠肿瘤细胞中发现草甘膦除草剂农达抑制参与性激素蛋白质生物合成活动。这把胆固醇 -- 妊娠烯醇酮 -- 孕激素转换途径的运行降低到最低水平。

2) Walsh, L.P. et al., (2000). Roundup inhibits steroidogenesis by distrupting

steroidogenic acute regulatory (StAR) protein expression. Environmental Health Perspectives, 108, 769-776.
Walsh, L.P. et al. (2000)。草甘膦除草剂农达通过干扰类固醇激素合成急性调节抑制(StAR)类固醇生成。环境健康前景，108, 769-776.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1638308/

科学证据 3（2002）：简要讲，农达通过草甘膦及其配方制剂的协同效应延迟 CDK1/细胞周期蛋白 B 的活动性影响细胞的生长周期。考虑到不同物种中 CDK1/细胞周期蛋白 B 调制器的普遍性，我们质疑草甘膦与农达对人类健康的安全性。

3) Marc J, Mulner-Lorillon O, Boulben S, Hureau D, Durand G, Bellé R. Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chem Res Toxicol. 2002;15(3):326-31.
http://www.ncbi.nlm.nih.gov/pubmed/11896679

科学证据 4（2004 年）：草甘膦除草剂抑制首个细胞周期的 G2/M 阶段的 DNA 生物合成。研究者们估计，草甘膦生产厂的工人吸入该项试验中浓度 500-5000 倍浓度的草甘膦。

4) Marc, J et al., (2004). Formulated glyphosate activities the DNA-response checkpoint of the cell cycle leading to the prevention of G2/M transition. Toxicological Sciences, 82, 436-442.
Marc, J et al., (2004)。草甘膦基配方除草剂激化细胞周期的 DNA 反应检查点导致阻止 G2/M 转变。毒理性科学，82, 436-442.
就职机构：细胞周期发展生物站、国家科学研究所、法国皮尔与玛丽居里大学
http://www.ncbi.nlm.nih.gov/pubmed/15375296

科学证据 5（2004）**：农业喷洒剂量稀释 500 至 4000 倍的草甘膦除草剂导致发展癌症的细胞周期机能失调。**

5) Marc J1, Mulner-Lorillon O, Bellé R. Glyphosate-based pesticides affect cell cycle regulation. Biol Cell. 2004 Apr;96(3):245-9.
http://www.ncbi.nlm.nih.gov/pubmed/15182708

科学证据 6（2005）：研究试验口服 1%浓度草甘膦在 21 天孕期中对怀孕鼠的血清与肝及其胎儿的脂质过氧化与抗氧化酶系统的影响。结果发现，摄入草甘膦诱发过量脂质过氧化，导致对怀孕鼠及其胎儿抗氧化防御系统过量。

6) Beuret CJ et al, Effect of the herbicide glyphosate on liver lipoperoxidation in pregnant rats and their fetuses.
Reprod Toxicol. 2005 Mar-Apr;19(4):501-4.
Beuret CJ et al,草甘膦除草剂在怀孕鼠及其胎儿中对肝脂质过氧化的影响。繁殖毒理学杂志。2005 年 3 月-4 月；19(4):501-4.
就职机构：阿根廷国立圣路易斯大学生物学与药理学系
http://www.ncbi.nlm.nih.gov/pubmed/15749264

科学证据 7（2005）：在哺乳动物中可以观察到草甘膦除草剂农达的内分泌干扰与毒性影响，不仅是草甘膦的内分泌干扰与毒性影响。我们认为农达的辅佐剂强化了的草甘膦的生物可获得性和/或生物积蓄。

7) Richard S et al., Differential effects of glyphosate and roundup on human placental cells and aromatase. Environ Health Perspect. 2005 Jun;113(6):716-20.
Richard S et al.，草甘膦与草甘膦除草剂浓度对人类胎盘细胞与芳香酶的差异性影响。环境性健康前景。2005 年 6 月；113(6):716-20.
就职机构：法国卡昂大学分子生物与生物化学实验室

http://www.ncbi.nlm.nih.gov/pubmed/15929894

科学证据 8（2007）：接触草甘膦除草剂造成对睾丸与附睾区域构造的改变，还改不了血清中睾丸激素与雌二醇的水平，改变了睾丸雄性激素受体的表达。更明显的近端传对微胆管和附睾导管造成有害影响，表明雄生殖器中这些部位对草甘膦更为敏感。造成的影响具有剂量依赖性，表明草甘膦除草剂可能在动物雄生殖系统中造成形态生理学失调。

8) Oliveira AG et al., Effects of the herbicide Roundup on the epididymal region of drakes Anas platyrhynchos. Reprod Toxicol. 2007 Feb;23(2):182-91. Epub 2006 Nov 11.
Oliveira AG et al.，草甘膦除草剂农达对绿头鸭附睾部位的影响。繁殖毒理学。2007 年 2 月；23(2):182-91。网络发表：2006 年 11 月 11 日。
就职机构：巴西 Minas Gerais 联邦大学形态学系
http://www.ncbi.nlm.nih.gov/pubmed/17166697

科学证据 9（2007）：研究结果表明，草甘膦除草剂农达没有诱发雌鼠毒性，但是对雄性后代造成了有害繁殖性影响：鼠仔成年后每个附睾精子数量与每天精子产生量减少、增加异常精子百分比，进入青春期后血清睾丸激素水平产生与草甘膦接触量相关减少，同时在两个阶段出现精子退化迹象。后代雌鼠仔长大后仅发生阴道开通延迟。这些发现表明，子宫内与哺乳期接触草甘膦除草剂农达可能对雄鼠仔进入青春期与成年期阶段的生殖系统诱发显著有害影响。

9) Dallegrave E et al., Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch Toxicol. 2007 Sep;81(9):665-73. Epub 2007 Jul 19.
Dallegrave E et al.商业草甘膦除草剂配方在 Wistar 鼠中的出生前与出生后毒性，毒理学档案。2007 年 9 月；81(9):665-73.网络出版：2007 年 7 月 19 日。

就职机构：巴西 Rio Grande do Sul 联邦大学，药理学系
http://www.ncbi.nlm.nih.gov/pubmed/17634926

科学证据 10（2009）：对草甘膦及其主要代谢物 AMPA，以及草甘膦添加除草剂配方中主要辅佐剂 POEA（表面活性剂，稀释 10 万倍，对三种不同的人类细胞的毒性。这样的稀释水平，远远低于农业应用推荐的水平，并对应于食品或者饲料中草甘膦残留的低水平。三种人类细胞分别为新生儿脐带静脉的细胞、293 胚肾细胞与 JEG3 胎盘细胞系。所有草甘膦的配方在 24 小时内造成所有细胞死亡，通过抑制线粒体琥珀酸脱氢酶活性，以及通过释放胞质膜伤害腺苷酸激酶测量膜损伤导致坏疽。通过激化酶的半胱天冬酶 3/7 活性诱发细胞凋亡。单独草甘膦激起仅细胞凋亡，而人脐静脉内皮细胞（HUVEC）在这个水平上 100 倍更敏感。有害效应与草甘膦浓度不成比例，更加取决于除草剂辅佐剂的性质。草甘膦代谢物 AMPA 与 POEA（草甘膦除草剂配方中的表面活性剂），分别单独作用或者合在一起协作用时，像草甘膦一样，损伤细胞膜，但是各自在不同的浓度发挥作用。它们与草甘膦一起的混合物的危害通常更强。结论，像 POEA 这样的辅佐剂，改变人类细胞的渗透性，以凋亡与坏疽方式强化诱发草甘膦诱发的毒性。草甘膦真正的阈值，必须考虑存在的辅佐剂，还必须考虑草甘膦的代谢、时间放大效应或生物蓄积作用。市场上销售的草甘膦除草剂，即便在抗草甘膦作物加工的食品与饲料残留水平，能够造成细胞损伤以至死亡。

10) Nora Benachour and Gilles-Eric Séralini, Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells, *Chem. Res. Toxicol.*, 2009, *22* (1), pp 97–105
Nora Benachour and Gilles-Eric Séralini，草甘膦配方除草剂在人类脐带、胚芽与胎盘细胞诱发凋亡与坏疽，化学研究毒理学，2009, *22* (1), pp 97–105
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全文：http://pubs.acs.org/doi/full/10.1021/tx800218n

科学证据 11（2009）：草甘膦为基础草甘膦在世界上最广泛使用。它们的残留成为环境中经常有的污染物。此外，这些除草剂还喷洒到最大量食用的转基因作物，这样的作物使其容忍细胞中高水平的这些成分。某些饲料中允许它们高达 400 ppm 残留。人类肝脏 HepG2 细胞是研究异型生物质毒性的知名模型，我们让人类肝脏 HepG2 细胞接触草甘膦及其四种不同配方除草剂制剂。通常仅在慢性活体内对单独草甘膦成分进行试验。我们用三种试验方法（Alamar Blue, MTT, ToxiLight），以及基因毒性（彗星试验）、抗雌激素（对 ERalpha, ERbeta）与抗雄激素效果（对 AR）做基因检测试验。我们还用芳香化酶活性与 mRNA 检测雄激素雌激素转换。所有指标在 24 小时内都受到亚农业用剂量用的草甘膦及其四种配方除草剂制剂所有成分的干扰。其效果更依赖于草甘膦除草剂的配方而非草甘膦的剂量。首先观察到的人类细胞内分泌干扰是最为活性配方制剂（R400）从 0.5 ppm 剂量在 MDA-MB453-kb2 细胞中对雄激素受体的作用，然后从 2 ppm 剂量起，HepG2 细胞的两个雌激素受体的转录活动性遭到抑制。从 10 ppm 剂量起，芳香化酶转录和活动收到干扰。在 Alamar Blue 试验（最敏感的）中，从 10 ppm 剂量其发生细胞毒性作用，但从 5 ppm 起发生 DNA 损伤。因此必须考虑食物、饲料或者环境中草甘膦除草剂对真实细胞的影响，对草甘膦分类为致癌物/致突变/致生殖毒性进行了讨论。

11) Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Séralini GE. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 2009;262:184-91. doi:10.1016/j.tox.2009.06.006.
Gasnier C, Dumont C, Benachour N, Clair E, Chagnon MC, Séralini GE。草甘膦除草剂在人类细胞系中致细胞毒性而且是内分泌干扰剂毒理学。2009;262:184-91.
http://www.ncbi.nlm.nih.gov/pubmed/19539684

科学证据 12（2010）：研究结果表明商业配方的草甘膦除草剂在体内是一种威力强大的内分泌干扰剂，青春期接触时对老鼠的生育系统发育造成干扰。

12) Romano RM et al., Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Arch Toxicol. 2010 Apr;84(4):309-17. Epub 2009 Dec 12.
Romano RM et al.，青春期前接触商业草甘膦除草剂改变睾丸激素水平与睾丸形态。毒理学档案。2010 年 4 月；84(4):309-17. 网络发表 2009 年 12 月 12 日。
就职机构：巴西 Sao Paulo 大学兽医学院，动物生殖与荷尔蒙实验室剂量系
http://www.ncbi.nlm.nih.gov/pubmed/20012598

科学证据 13（2010）：用 1ppm 浓度草甘膦对沙漏树蛙蝌蚪处理后，近 60%发育失调。

13) Jayawardene, U.A et al., (2010). Toxicity of agrochemicals to common hourglass tree frog (Polypedates crugiger) in acute and chronic exposure. International Journal of Agriculture and Biology, 12, 641-648.
Jayawardene, U.A et al., (2010)。农业化学品急性与慢性接触对普遍的沙漏树蛙（Polypedates crugiger）的毒性。国际农业与生物学杂志，12, 641-648.
http://www.fspublishers.org/ijab/past-issues/IJABVOL_12_NO_5/1.pdf

科学证据 14（2010）：非洲爪蟾蜍晶胚与稀释 5000 倍草甘膦除草剂一起孵化。经处理的晶胚高度异常，头盖与神经嵴明显改变，前后轴缩短。改变后神经嵴标记与颅软骨蝌蚪阶段畸形一致。注射单独草甘膦显示非常类似的畸形。草甘膦除草剂在鸡晶胚中显示类似的影响，显示一个逐渐失去了菱域。减少视觉囊泡和小头畸形。这表明草甘膦自己对观察到的畸形负责，而不是草甘膦除草剂配方中的表面活性剂或其他组分。一个报告基因分析，揭示草甘膦除草剂处理增加了非洲爪蟾蜍晶胚中的视黄酸活性，而且与 RA 拮抗剂的协作处理保持了草甘膦除草剂的致畸效应。因此结论草甘膦除草剂产生的显型主要是内源性类活动增加的结果。

14) Paganelli, A.et al., (2010). Glyphosate- based herbicides produce teratogenic effects on vertebrates by impairing retionic acid signaling. Chemical Research in Toxicology, 23, 1586-1595.
Paganelli, A.et al., (2010)。草甘膦基除草剂通过损伤损害视黄酸信号对脊椎动物造成畸形影响。毒理学化学研究，23, 1586-1595.
就职机构：阿根廷布宜诺斯艾利斯大学医学院胚胎分子学实验室
http://pubs.acs.org/doi/abs/10.1021/tx1001749

科学证据 15（2012）：草甘膦除草剂是世界最大量销售的除草剂；最普遍的配方制剂农达含 POEA 作为其主要表面活性剂。最新的发现表明，暴露于草甘膦对人类可能造成 DNA 损伤与癌症。 ...与早期的草甘膦对内部器官淋巴与细胞的研究进行比较表明，上皮细胞更容易受到细胞毒性，而且 DNA 损伤的性质与除草剂及其配方相关。由于我们发现农业中喷洒剂量稀释 450 倍的短期暴露造成基因毒性的影响，我们的发现表明，喷洒草甘膦除草剂可能造成吸入人类 DNA 损伤。

15) Koller VJ, Furhacker M, Nersesyan A, Misik M, Eisenbauer M, Knasmueller S. Cytotoxic and DNA-damaging properties of glyphosate and Roundup in human-derived buccal epithelial cells. Arch Toxicol. 2012;86:805–813. doi:10.1007/s00204-012-0804-8.
Koller VJ, Furhacker M, Nersesyan A, Misik M, Eisenbauer M, Knasmueller S. 草甘膦与农达在人类口腔上皮细胞中造成细胞毒性与 DNA 损伤的性质。毒理学档案。2012;86:805–813.
http://link.springer.com/article/10.1007%2Fs00204-012-0804-8

科学证据 16（2013）“该研究演示所试验的所有草甘膦为基础的除草剂都比单独草甘膦更为毒性……配方除草剂（包括农达）可以影响所有活的细胞，特别人类细胞。在它们之中，（农达配方中的表面活性剂）POE-15 清楚显示是对人类细胞最为毒性的成分…… 除了 POE-15 在它的首次胶束

化作用发生时诱发坏疽之外，这与草甘膦进入系背后促进内分泌干扰作用有所不同。”

16) R. Mesnagea, B. Bernayc, G.-E. Séralinia, Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity, Toxicology， Volume 313, Issues 2–3, 16 November 2013, Pages 122–128
http://www.sciencedirect.com/science/article/pii/S0300483X12003459

科学证据 17（2013）：草甘膦是最为广泛使用并被人们相信比其他农药毒性较低的除草剂的活性成分。然而，最近几项研究显示它对人类潜在危害健康，而且可能是一种内分泌干扰剂。该项研究集中于纯草甘膦对雌激素受体蛋白（**estrogen receptors**）促进的转录活动性及其表达。草甘膦，在 **10^{-12}** 至 **10^{-6} M**（万亿分之一至百万分之一质量）范围，在雌激素撤走状态下仅对人类激素**-**依赖乳房癌细胞 **T47D** 细胞发挥增殖作用，而对激素**-**独立乳房癌细胞 **MDA-MB231** 细胞系没有这种作用。诱发 **ERE**（雌激素反应元素）转录活动性达到 **T47D-KBluc** 细胞中对照组的 **5-13** 倍的产生增殖作用剂量的草甘膦，受到一种雌激素对抗药 **ICI 182780** 的抑制，表明草甘膦的雌激素活动性通过雌激素受体蛋白（**estrogen receptors**）发挥作用。此外，草甘膦既改变雌激素受体蛋白（**estrogen receptors**）**α**，有改变其 **β** 表达。这些结果显示，在低的与环境性相关浓度下，草甘膦具有雌激素性活动。草甘膦为基础的除草剂广泛用于（转基因）大豆种植，而我们的结果发现，存在着草甘膦与大豆中的一种植物雌激素染料木黄酮（**genistein**）之间的额外雌激素作用。然而，草甘膦对于（转基因）大豆的这种额外的作用需要进行进一步的动物试验。

17) Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J. Glyphosate induces human breast cancer cells growth via estrogen receptors. Food Chem Toxicol. 2013. doi:10.1016/j.fct.2013.05.057.

Thongprakaisang S, Thiantanawat A, Rangkadilok N, Suriyo T, Satayavivad J.
草甘膦通过雌激素受体蛋白诱发人类乳房癌细胞生长。食物化学毒理学。2013 年 6 月 9 日发布
http://www.ncbi.nlm.nih.gov/pubmed/23756170

Decline in semen quality among 30,636 young Chinese men from 2001 to 2015

Chuan Huang, Ph.D.,[a] Baishun Li, B.S.,[b] Kongrong Xu, M.S.,[a] Dan Liu, M.S.,[a] Jing Hu, M.S.,[b] Yang Yang, B.S.,[b] HongChuan Nie, M.D., Ph.D.,[b] Liqing Fan, M.D., Ph.D.,[a,b] and Wenbing Zhu, M.D., Ph.D.[a,b]

[a] Institute of Reproductive and Stem Cell Engineering, Basic Medicine College, Central South University; and [b] Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha, Hunan, People's Republic of China

Objective: To provide information of semen quality among young Chinese men in the past 15 years.
Design: Retrospective cross-sectional study.
Setting: Sperm bank.
Patient(s): A total of 30,636 young adult men who applied to be sperm donors at the Hunan Province Human Sperm Bank of China in 2001–2015 were included in the study.
Intervention(s): Physical examination and analysis of blood and semen samples.
Main Outcome Measure(s): Semen parameters, such as semen volume, sperm concentration, total sperm count, progressively motile sperm count, sperm progressive motility, sperm morphology, and round cells.
Result(s): Many of the semen parameters showed a decreasing trend over the 15-year observation period. The sperm concentration and percentage of sperm with normal morphology decreased from 68×10^6/mL to 47×10^6/mL and from 31.8% to 10.8%, respectively. Although sperm progressive motility showed irregular variation, the progressively motile sperm count decreased from 34×10^6 to 21×10^6 over the 15-year period. Furthermore, the rate of qualified donors fell from 55.78% in 2001 to 17.80% in 2015, and the rate for 2015 was approximately threefold lower than the corresponding rates in 2001.
Conclusion(s): The semen quality among young Chinese men has declined over a period of 15 years, especially in terms of sperm concentration, total sperm count, sperm progressive motility, and normal morphology. (Fertil Steril® 2017;107:83–8. ©2016 by American Society for Reproductive Medicine.)
Key Words: Chinese young men, semen parameters, semen quality

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Semen quality is related to the quality of reproductive health and is a very important factor that reflects male reproductive health. In recent years, numerous reports have indicated that the semen quality in normal men is declining. In the early 1990s, Carlsen et al. (1) reviewed more than 60 papers worldwide and found a trend of decreasing sperm count and seminal fluid volume over the past 50 years. Many researchers were skeptical about the results, and several researchers were prompted to study trends in their own countries. Swan et al. (2) reviewed 101 studies in the literature and verified that there was indeed a decline in sperm count over time. Likewise, Huang et al. (3) reviewed 115 studies from 1985 to 2009 reporting the manual sperm counts of 23,126 healthy Chinese men and reported a possible decline in the semen concentration over that 25-year period. Papers reported heterogeneous findings, with some studies confirming a decreasing trend in semen quality while others did not. For example, Jorgensen et al. (4) showed an increasing trend in sperm concentration and total sperm count in 4,867 young men in Copenhagen, Denmark. Similarly, Zhu et al. (5) reviewed 36 papers and analyzed the semen parameters from 2,318 healthy Chinese men and showed no significant decline in sperm density and semen volume over a 13-year period. Despite differences, several studies have reported relatively poor semen quality in their study populations (6–8), and

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Reprint requests: Wenbing Zhu, M.D., Ph.D., Reproductive and Genetic Hospital of CITIC-Xiangya, No. 87, Xiangya Road, Changsha, Hunan 410008, People's Republic of China (E-mail: zhuwenbing0971@sina.com).

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very few long-term studies on the trends in semen quality have been carried out in the same laboratory in China.

To our knowledge, no long-term studies on semen quality in the Chinese population have been published so far. The present study aimed to investigate whether the semen parameters in young Chinese men have declined over the past 15 years. To this end, we retrospectively reviewed the semen parameters of a population of young adult men applying for consideration as sperm donors in Hunan, China, from 2001 to 2015.

MATERIALS AND METHODS

Study Population

In this retrospective study, we reviewed the semen analysis database of young adult men who applied to be sperm donors at the Hunan Province Human Sperm Bank of China from August 30, 2001, to December 31, 2015. Relevant demographic and clinical information of all of the men was collected and analyzed. Demographic information included age, height, weight, smoking and drinking history, and duration of abstinence. Clinical information included semen parameters and the date of semen analysis.

All donors signed informed consent forms during their first visit to the human sperm bank, agreeing that their semen samples or data could be used by the human sperm bank for scientific research. The present study was approved by the Ethics Committee of the Reproductive and Genetic Hospital of CITIC-Xiangya (LL-SC-SG-2015-003).

Semen Analysis

A total of 71,353 specimens from 30,636 young adult men were included in the analysis. Specimens were collected by means of masturbation into a sterile container after 2–7 days of abstinence. All specimens were assessed according to the World Health Organization (WHO) 1999 recommendations (9). After liquefaction and within 1 hour of ejaculation, the samples were analyzed for semen volume and sperm concentration, round cells, normal morphology, and sperm motility (defined as WHO motility grades A, B, C, and D, where grade A indicates fast progressive sperm, B slow progressive sperm, C nonprogressive sperm, and D immotile sperm). Sperm morphology was evaluated by means of the modified Papanicolaou staining method, and apart from the normal forms, any defects in the head, midpiece, and tail were recorded.

Criteria for Screening Sperm Donors in China

The recruitment methods for sperm donors include handing out leaflets, conducting lectures in schools, and network publicity by technicians. The screening of sperm donors is conducted in strict accordance with the standard guidelines published by the Chinese Ministry of Health in 2003. The guidelines are as follows (10): 1) Donors must be from 22 to 44 years of age; 2) donors must have a college degree or above, and height not less than 1.70 m; 3) donors must be in good health, based on the results of both physical examination and psychologic evaluation by qualified doctors, and have no familial history of genetic disease; 4) fresh semen should have a liquefaction time of <60 minutes, sperm concentration $\geq 60 \times 10^6$/mL, progressive sperm motility of $\geq$60%, and percentage of normal morphology >30%; 5) post-thaw semen should have a motility of $\geq$40%, $\geq 12 \times 10^6$ motile sperm, and a frozen-thaw survival rate of $\geq$60%; and 6) potential donors must undergo laboratory testing to exclude individuals at high risk for sexually transmitted infections and genetic diseases, including human immunodeficiency virus 1 and 2, hepatitis B and C, syphilis, gonorrhea, mycoplasma, chlamydia, cytomegalovirus, *Toxoplasma gondii*, rubella virus, herpes simplex virus types 1 and 2, and karyotype analysis. If the patient tests negative for all of the above tests and fulfills the Chinese Ministry of Health guidelines outlined above, the donation process is initiated and the semen samples are cryopreserved. The samples must be cryopreserved for a minimum 6-month quarantine period before rescreening for HIV. The way of recruiting and criteria for screening sperm donors have not been changed over time.

Statistical Analysis

Because semen parameters follow markedly skewed (nonnormal) distributions, unadjusted mean and median values, standard deviation (SD), and 5th to 95th percentiles were calculated for each variable. Percentages coinciding with WHO recommendations (1999, 2010) (9, 11) were also calculated. The study subjects were divided into three groups depending on the investigation periods: 2001–2005, 2006–2010, and 2011–2015. Between-group differences for continuous variables were tested by means of the nonparametric Kruskal-Wallis test. Statistical data were analyzed with the use of the Statistical Package for the Social Sciences (SPSS) 18.0. A P value of $<.05$ was considered to be statistically significant.

RESULTS

Subject Characteristics

The general demographic characteristics of the 30,636 men (including 3,114, 10,386, and 17,136 in the 2001–2015, 2006–2010, and 2011–2015 groups, respectively) are summarized in Table 1. No differences were found between the three groups in age (P=.79), height, weight, body mass index, abstinence times, and alcohol drinking and smoking habits over the study period.

Semen Parameters

The semen parameters of the study subjects are described in Table 2. As presented in the table, the semen volume and total count were within the high-normal values (82.3% and 78.2%, respectively, according to the 1999 WHO criteria and 89.6% and 85.4% according to the 2010 WHO criteria). However, the semen parameters, especially sperm progressive motility, among a large proportion of the study subjects were below the lower threshold of the WHO criteria. Additionally, only 50.7% of the semen samples had normal semen parameters according to the WHO 2010 criteria. At least one parameter in ~49.3% and 58.9% of the semen samples was below the

TABLE 1

Demographic characteristics of participants.

Characteristic	2001–2005 (n = 3,114) Mean (SD)	2001–2005 (n = 3,114) Median (5th–95th %ile)	2006–2010 (n = 10,386) Mean (SD)	2006–2010 (n = 10,386) Median (5th–95th %ile)	2011–2015 (n = 17,136) Mean (SD)	2011–2015 (n = 17,136) Median (5th–95th %ile)	Difference among the three groups (*P* value)
Age (y)	21.6 (3.1)	21.0 (19.0–24.0)	21.4 (2.3)	21.0 (19.0–24.0)	21.9 (2.8)	21.0 (19.0–28.0)	.79
Height (m)	1.72 (0.04)	1.72 (1.66–1.80)	1.72 (0.04)	1.72 (1.65–1.80)	1.73 (0.04)	1.72 (1.65–1.81)	.27
Weight (kg)	62.6 (7.0)	62.0 (53.0–74.0)	62.8 (8.9)	62.0 (52.0–75.0)	63.8 (8.3)	63.0 (52.0–80.0)	.53
Body mass index (kg/m^2)	21.1 (2.0)	20.9 (18.4–24.2)	21.1 (5.2)	20.8 (18.2–24.6)	21.3 (2.4)	21.0 (18.0–26.0)	.38
Abstinence (d)	4	4 (2–7)	4	3 (2–7)	4	3 (2–7)	.46
Smokers (%)	7.7		8.2		7.9		.25
Drinkers (%)	36.5		34.7		32.8		.07

Huang. Declining semen quality in Chinese men. Fertil Steril 2016.

normal threshold value according to the WHO 2010 and 1999 criteria, respectively.

Changes in Semen Parameters

Table 3 and Supplemental Figure 1 (available online at www.fertstert.org) present the changes in the semen parameters among the study participants. The semen parameters exhibited a decreasing trend over the past 15 years. The semen volume and round cells did not significantly differ among the three groups (P=.07 and P=.36, respectively). However, the sperm concentration, total sperm count, and normal sperm morphology decreased and the sperm progressive motility showed erratic changes over the 15-year observation period. The sperm concentration and percentage of sperm with normal morphology were decreased from 68 $\times$ 10^6/mL to 47 $\times$ 10^6/mL and from 31.8% to 10.8%, respectively, whereas the sperm progressive motility showed irregular variations, decreasing from 34 $\times$ 10^6 to 21 $\times$ 10^6 over 15 years.

Donors

As presented in Table 4, the percentage of qualified donors also exhibited a decreasing trend over the 15-year period, from 55.78% in 2001 to 17.80% in 2015. The percentage of qualified donors in 2015 was approximately threefold lower than in 2001 and declined by ~40% in the past 15 years. The main reasons for nonrecruitment was unacceptable semen parameters (97.1%, 19,285/19,865), including low sperm concentration (81.9%, 16,272/19,865), followed by low sperm motility, low semen volume, azoospermia, and hematospermia. Approximately 1.6% (319/19,865) of unqualified candidates tested positive for transmitted diseases, and a minority of patients could not be recruited owing to physical examination abnormalities and hereditary or chromosomal disorders (Supplemental Table 1, available online at www.fertstert.org).

DISCUSSION

The analysis of semen includes tests for semen volume, sperm concentration, sperm motility, and morphology. Although alternate tests based on more functional aspects, such as sperm penetration, capacitation, and acrosome reaction have been developed, semen analysis continues to be used as the primary method to determine male fertility, and it plays an important role in andrology. In the present study, we screened and analyzed 71,353 specimens from 30,636 healthy Chinese men. To our knowledge, this is the largest study focusing on the semen quality of young men from the general

TABLE 2

Summary of semen parameters.

Parameter	n	Mean (SD)	Median	Percentile 5th	Percentile 25th	Percentile 75th	Percentile 95th	Normal semen parameters according to the 1999 WHO recommendations (%)[a]	Normal semen parameters according to the 2010 WHO recommendations (%)[a]
Semen volume (mL)	30,636	2.6 (1.1)	2.3	0.8	1.5	3.0	4.5	82.3	89.6
Sperm concentration (million/mL)	30,636	53.4 (31.7)	50.0	11	35	68	93	69.7	81.9
Total sperm count (million)	30,636	127 (68)	130	13	75	198	267	78.2	85.4
Sperm progressive motility (a + b) (%)	30,476[b]	47.5 (22.1)	46	24	38	55	66	43.3	60.8
Normal sperm morphology (%)	30,476[b]	17.2 (8.7)	15.8	3.1	9.3	24.8	34.5	58.5	79.1

[a] Abnormal values of semen parameters were defined by the World Health Organization (WHO) recommendations (1999 and 2010). The 1999 standards: semen volume <2 mL, sperm concentration <20 $\times$ 10^6/mL, sperm total count <40 $\times$ 10^6, sperm progressive motility <50%, and normal morphology $\leq$15%. The 2010 standards: semen volume <1.5 mL, sperm concentration <15 $\times$ 10^6/mL, sperm total count <39 $\times$ 10^6, sperm progressive motility <32%, and normal morphology $\leq$4%.
[b] Number of sperm without azoospermia.

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TABLE 3

Semen quality of 30,636 young men from the general population in Hunan, China.

Variable	2001–2005 Mean (SD)	2001–2005 Median (5th–95th %ile)	2006–2010 Mean (SD)	2006–2010 Median (5th–95th %ile)	2011–2015 Mean (SD)	2011–2015 Median (5th–95th %ile)	Difference among the three groups (*P* value)
Semen volume (mL)	2.8 (1.1)	3.0 (1.5–4.5)	2.6 (1.1)	2.3 (0.8–4.5)	2.5 (1.2)	2.3 (0.8–4.5)	.07
Sperm concentration (million/mL)	68 (36)	64 (18–130)	58 (32)	60 (12–110)	47 (25)	50 (10–80)	.00
Total sperm count (million)	182 (69)	177 (22–338)	144 (67)	137 (9–297)	119 (74)	114 (13–236)	.00
Progressive motile sperm count (a + b) (million)	34 (20)	31 (7–71)	27 (19)	24 (5–55)	21 (35)	20 (1–39)	.00
Sperm progressive motility (a + b) (%)	50.2 (17.2)	51.6 (25.0–70.8)	43.1 (22.9)	44.8 (24.5–65.2)	47.1 (36.2)	46.0 (25.3–66.4)	.04
Round cells (million)	0.5 (0.4)	0.7 (0.1–3.0)	0.6 (0.9)	0.6 (0.1–2.0)	0.5 (0.7)	0.6 (0.2–2.7)	.36
Normal sperm morphology (%)	31.8 (6.4)	31.0 (22.0–42.0)	20.5 (7.4)	20.1 (11.4–39.4)	10.8 (6.7)	10.6 (2.5–34.6)	.00
Normal semen parameters according to the 1999 WHO recommendations (%)	66.3		47.1		32.9		.00
Normal semen parameters according to the 2010 WHO recommendations (%)	76.7		56.5		42.4		.00

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population in China and is the first to report the long-term semen quality trends within a single laboratory in China.

We analyzed the semen quality from 30,636 young men from Hunan Province, China. Our findings were not in complete agreement with the semen parameters observed in several other studies in young Chinese men. The semen quality of the young Chinese men in our study was not optimal, although the mean and median values of semen volume in our study (2.6 and 2.3 mL, respectively) were similar to those reported in previous studies in China (12–17). The mean sperm concentration and total sperm count in our study (53.4×10^6/mL and 127×10^6, respectively) were markedly lower than those reported previously, ranging from 50.2–84.8 $\times 10^6$/mL and from 124.1–206.9 $\times 10^6$, respectively. The mean normal sperm morphology in our study (15.8%) was also observably lower than in some of the previous studies, where it ranged from 9.5% to 68%. The discrepancies between the previous studies and ours may be because previous studies included young men with a broader age range compared with the participants in our study. The mean sperm progressive motility in our study (47%) was similar to that reported in other studies in young Chinese men. In our study, the semen parameters of only 41.1% of the specimens were completely normal according to the 1999 WHO criteria. More recently, WHO revised the semen reference values in 2010 by studying the semen parameter distributions of men whose partners had a time-to-pregnancy (TTP) of up to and including 12 months; these new WHO criteria (2010) are lower than previous WHO criteria (1999) (18, 19). However, even when the revised (2010) WHO standards were used as reference, semen parameters in only 50.7% of the study participants were all within the normal range. A higher proportion of men in countries had abnormal semen parameters than Chinese men. The mean values of all semen parameters were lower in our study than in studies in other countries in general (4,20–23), especially regarding the mean semen volume, which was lower in Chinese men by 0.6–1.4 mL. The reasons for the reported differences in the semen quality between our study and previously reported studies remain to be understood. It is possible that geographic variations are responsible for the observed differences in semen quality, and these regional variations result from different interactions among lifestyle, other environmental factors, and genetic variations, or a combination of these factors.

Since the founding of the Human Sperm Bank, all of the technicians working in the laboratory have received the same training. All semen samples were analyzed by five well trained laboratory technicians with the use of the same apparatus, and efforts were made to keep the technique of semen analysis unchanged. As such, the change in semen parameters

TABLE 4

Percentage of qualified sperm donors from 2001 to 2015.

Year	New sperm donors, n	Qualified donors, n	Qualified donors, %
2001	95	53	55.78
2002	366	193	52.73
2003	598	314	52.51
2004	803	409	50.93
2005	1,252	611	48.80
2006	1,564	718	45.91
2007	2,244	1,096	48.84
2008	1,925	781	40.57
2009	2,107	979	46.46
2010	2,546	965	37.90
2011	2,628	849	32.31
2012	2,133	823	38.56
2013	3,031	1,019	33.61
2014	4,523	1,103	24.39
2015	4,821	858	17.80

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can not be attributed to variations in laboratory technique or laboratory technicians. And internal quality control measures were performed to avoid drift over time.

The debate on declining semen quality is still ongoing (24). In contrast to the findings of the present study, Jørgensen et al. (4) reported that semen quality among young Danish men from the general population of Denmark showed an increasing trend in sperm concentration and total sperm count from 1996 to 2010. Our study clearly indicated that sperm concentration, sperm count, motility, and normal morphology of sperm showed a significant and continuous decrease over the 15-year observation period. This declining tendency was constant throughout but was more evident for sperm concentration. Centola et al. (25) reported a decline in sperm count and motility among young adult men in the Boston area during the past 10 years. Mendiola et al. (26) suggested that the total sperm count and sperm concentration may have declined in young southern Spanish men from 2001 to 2011. Similarly, Rolland et al. (27) showed an annual decrease of 1.9% in the sperm concentration, a significant but not quantifiable decrease in morphologically normal sperm, and a significant increase in total motility over a 17-year period in their study population in France. As presented in Table 4 and Supplemental Table 1, the rate of qualified donors showed a decreasing trend, with the main reason for nonrecruitment being unacceptable semen parameters. Together, these findings indicated that semen quality showed a significant and continuous declining trend over the 15-year study period. On the other hand, a recent comprehensive review of the 1992 meta-analysis and subsequent reports suggested that sperm counts are not declining (28). However, the data reported in the present study are particularly relevant, because the global decline in semen quality may lead to an increase in the numbers of men falling into the subfertile range in terms of semen parameters (29). Indeed, there has been an increasing opinion that the criteria and reference ranges for screening sperm donors in China should be reconsidered.

In the present study, we analyzed the semen quality in a regional homogeneous population, apparently living under the same climatic and environmental conditions. However, the reasons for the decline in semen parameters are unclear from the present study. Some studies (30, 31) have shown that smoking and alcohol consumption have a negative effect on semen parameters, but in the present study there was no significant difference in the semen parameters between men who smoked or drank alcohol and those who did not over the 15 years; therefore, we speculated that there may have been risk factors besides smoking and alcohol consumption that affected the semen quality. Over the past two decades, there has been a rapid pace of economic and social change in China, and this has been followed by increased environmental pollution, including pollution of water, air, and food. This pollution has been reported to have high levels that may alter men sperm quality (32–34). Therefore, we speculated that pollution may be one of the causes of the decline in semen quality. In addition, nowadays, young men experience greater psychologic stress from study, work, and emotional problems, which also adversely affect semen quality (35). Lifestyle changes are another key factor that should not be ignored, and an increasing number of reports have confirmed that the widespread use of mobile phones and wireless technologies by young men has an impact on sperm quality. Yildirim et al. (36) indicated a negative correlation between wireless internet and mobile phone use duration and total sperm count. Similarly, Wang et al. (37) found that mobile phone radiation reduces the progressive motility and viability of human sperm and increases sperm head defects and early apoptosis of sperm cells. In addition, irregular living habits of young men, including staying up late, playing computer games, and staying overnight in bars, also can cause a decline in sperm quality (17). There is no doubt that the reasons for the decline in semen parameters are complex and can include factors such as environmental pollution, increased stress, and lifestyle. More data and statistical analyses are required to study the risk factors for decline in sperm quality.

Limitations of the present study include the lack of questionnaire data (e.g., history of diagnosis and previous treatment received) from the young adult men who participated in this study; therefore, we can not provide strong evidence regarding the influence of various risk factors on semen quality (4). Furthermore, our findings may not be based on a community population, because the study group was young and with a limited age range. This study population represents only one geographical area of China, and may not be representative of China as a whole. More studies of this type are needed.

This is the first study to investigate the semen quality of a large population within the same laboratory in China over a long observation period. Our data clearly illustrate that the semen quality in young men in China has been declining over the past 15 years, especially in terms of the sperm concentration, total sperm count, sperm progressive motility, and normal morphology. Moreover, the percentage of qualified donors also showed a decreasing trend during this time period. Although bulk semen parameters (reflected by 95% confidence intervals) overlap substantially throughout the study period, overall, these findings are a serious reproductive health warning, and further studies are warranted to confirm the findings of this study in China and to determine the factors causing this phenomenon.

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REFERENCES

1. Carlsen E, Giwercman A, Keiding N, Skakkebaek NE. Evidence for decreasing quality of semen during past 50 years. BMJ 1992;305:609–13.
2. Swan SH, Elkin EP, Fenster L. The question of declining sperm density revisited: an analysis of 101 studies published 1934–1996. Environ Health Perspect 2000;108:961–6.
3. Huang LP, Li YF, Xiong HY, Cao J. Trend of change in semen quality in Chinese healthy men over recent 25 years. Reprod Contracept 2011;2:122–39.
4. Jorgensen N, Joensen UN, Jensen TK, Jensen MB, Almstrup K, Olesen IA, et al. Human semen quality in the new millennium: a prospective cross-

sectional population-based study of 4867 men. BMJ Open 2012;2: e000990.
5. Zhu XQ, Shen Y, Zhang XY, Chang Y, Huang X. Trend Analysis of Sperm Quality During the Past 13 Years in China. J Zhejiang Univ 2000;29:173–6.
6. Bonde JP, Ernst E, Jensen TK, Hjollund NH, Kolstad H, Henriksen TB, et al. Relation between semen quality and fertility: a population-based study of 430 first-pregnancy planners. Lancet 1998;352:1172–7.
7. Guzick DS, Overstreet JW, Factor-Litvak P, Brazil CK, Nakajima ST, Coutifaris C, et al. Sperm morphology, motility, and concentration in fertile and infertile men. N Engl J Med 2001;345:1388–93.
8. Slama R, Eustache F, Ducot B, Jensen TK, Jorgensen N, Horte A, et al. Time to pregnancy and semen parameters: a cross-sectional study among fertile couples from four European cities. Hum Reprod 2002;17:503–15.
9. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 4th ed. 1999.
10. Ping P, Zhu WB, Zhang XZ, Li YS, Wang QX, Cao XR, et al. Sperm donation and its application in China: a 7-year multicenter retrospective study. Asian J Androl 2011;13:644–8.
11. World Health Organization. WHO laboratory manual for the examination and processing of human semen. 5th ed. 2010.
12. Junqing W, Qiuying Y, Jianguo T, Wei Y, Liwei B, Yuxian L, et al. Reference value of semen quality in Chinese young men. Contraception 2002;65:365–8.
13. Liu M, Ma C, Tang L, Wen R, Deng S, Wang Q, et al. [Quality analysis of the primary semen samples from 512 donors]. Zhonghua Nan Ke Xue 2004;10: 734–6.
14. Gao J, Gao ES, Walker M, Yang Q, Wu JQ, Zhu QX, et al. Reference values of semen parameters for healthy Chinese men. Urol Int 2008; 81:256–62.
15. Li Y, Lin H, Ma M, Li L, Cai M, Zhou N, et al. Semen quality of 1346 healthy men, results from the Chongqing area of southwest China. Hum Reprod 2009;24:459–69.
16. Jiang M, Chen X, Yue H, Xu W, Lin L, Wu Y, et al. Semen quality evaluation in a cohort of 28213 adult males from Sichuan area of south-west China. Andrologia 2014;46:842–7.
17. Rao M, Meng TQ, Hu SH, Guan HT, Wei QY, Xia W, et al. Evaluation of semen quality in 1808 university students, from Wuhan, Central China. Asian J Androl 2015;17:111–6.
18. World Health Organization. Laboratory manual for the examination of human semen and semen–cervical mucus interaction. Geneva: World Health Organization; 1999.
19. Cooper TG, Noonan E, von Eckardstein S, Auger J, Baker HW, Behre HM, et al. World Health Organization reference values for human semen characteristics. Hum Reprod Update 2010;16:231–45.
20. Axelsson J, Rylander L, Rignell-Hydbom A, Giwercman A. No secular trend over the last decade in sperm counts among Swedish men from the general population. Hum Reprod 2011;26:1012–6.
21. Mendiola J, Jorgensen N, Andersson AM, Stahlhut RW, Liu F, Swan SH. Reproductive parameters in young men living in Rochester, New York. Fertil Steril 2014;101:1064–71.
22. Jorgensen N, Vierula M, Jacobsen R, Pukkala E, Perheentupa A, Virtanen HE, et al. Recent adverse trends in semen quality and testis cancer incidence among Finnish men. Int J Androl 2011;34:e37–48.
23. Iwamoto T, Nozawa S, Mieno MN, Yamakawa K, Baba K, Yoshiike M, et al. Semen quality of 1559 young men from four cities in Japan: a cross-sectional population-based study. BMJ Open 2013;3.
24. Andersson AM, Jorgensen N, Main KM, Toppari J, Rajpert–de Meyts E, Leffers H, et al. Adverse trends in male reproductive health: we may have reached a crucial "tipping point". Int J Androl 2008;31:74–80.
25. Centola GM, Blanchard A, Demick J, Li S, Eisenberg ML. Decline in sperm count and motility in young adult men from 2003 to 2013: observations from a U.S. sperm bank. Andrology 2016;4:270–6.
26. Mendiola J, Jorgensen N, Minguez-Alarcon L, Sarabia-Cos L, Lopez-Espin JJ, Vivero-Salmeron G, et al. Sperm counts may have declined in young university students in southern Spain. Andrology 2013;1:408–13.
27. Rolland M, Le Moal J, Wagner V, Royere D, de Mouzon J. Decline in semen concentration and morphology in a sample of 26,609 men close to general population between 1989 and 2005 in France. Hum Reprod 2013;28:462–70.
28. Pastuszak AW, Lamb DJ. Counting your sperm before they fertilize: are sperm counts really declining? Asian J Androl 2013;15:179–83.
29. Murray KS, James A, McGeady JB, Reed ML, Kuang WW, Nangia AK. The effect of the new 2010 World Health Organization criteria for semen analyses on male infertility. Fertil Steril 2012;98:1428–31.
30. Pourentezari M, Talebi AR, Mangoli E, Anvari M, Rahimipour M. Additional deleterious effects of alcohol consumption on sperm parameters and DNA integrity in diabetic mice. Andrologia 2016;48:564–9.
31. Sharma R, Harlev A, Agarwal A, Esteves SC. Cigarette smoking and semen quality: a new meta-analysis examining the effect of the 2010 World Health Organization laboratory methods for the examination of human semen. Eur Urol 2016.
32. Fathi Najafi T, Latifnejad Roudsari R, Namvar F, Ghavami Ghanbarabadi V, Hadizadeh Talasaz Z, Esmaeli M. Air pollution and quality of sperm: a meta-analysis. Iran Red Crescent Med J 2015;17:e26930.
33. Deng Z, Chen F, Zhang M, Lan L, Qiao Z, Cui Y, et al. Association between air pollution and sperm quality: a systematic review and meta-analysis. Environ Pollut 2016;208:663–9.
34. Adamopoulos DA, Pappa A, Nicopoulou S, Andreou E, Karamertzanis M, Michopoulos J, et al. Seminal volume and total sperm number trends in men attending subfertility clinics in the greater Athens area during the period 1977–1993. Hum Reprod 1996;11:1936–41.
35. Gollenberg AL, Liu F, Brazil C, Drobnis EZ, Guzick D, Overstreet JW, et al. Semen quality in fertile men in relation to psychosocial stress. Fertil Steril 2010;93:1104–11.
36. Yildirim ME, Kaynar M, Badem H, Cavis M, Karatas OF, Cimentepe E. What is harmful for male fertility: cell phone or the wireless internet? Gaoxiong Yi Xue Ke Xue Za Zhi 2015;31:480–4.
37. Wang D, Li B, Liu Y, Ma YF, Chen SQ, Sun HJ, et al. [Impact of mobile phone radiation on the quality and DNA methylation of human sperm in vitro]. Zhonghua Nan Ke Xue 2015;21:515–20.

SUPPLEMENTAL TABLE 1

Distribution of 19,865 sperm donors based on reason for exclusion.

Reason for exclusion	n	%
Sperm concentration <60 × 10^6/mL, motility <60%	8,882	44.7
Sperm concentration <60 × 10^6/mL, motility >60%	7,390	37.2
Sperm concentration >60 × 10^6/mL, motility <60%	2,227	11.2
Azoospermia	160	0.8
Semen volume <2 mL	626	3.2
Sexually transmitted diseases	319	1.6
Hereditary or chromosomal disorders	138	0.7
Physical examination abnormality	123	0.6

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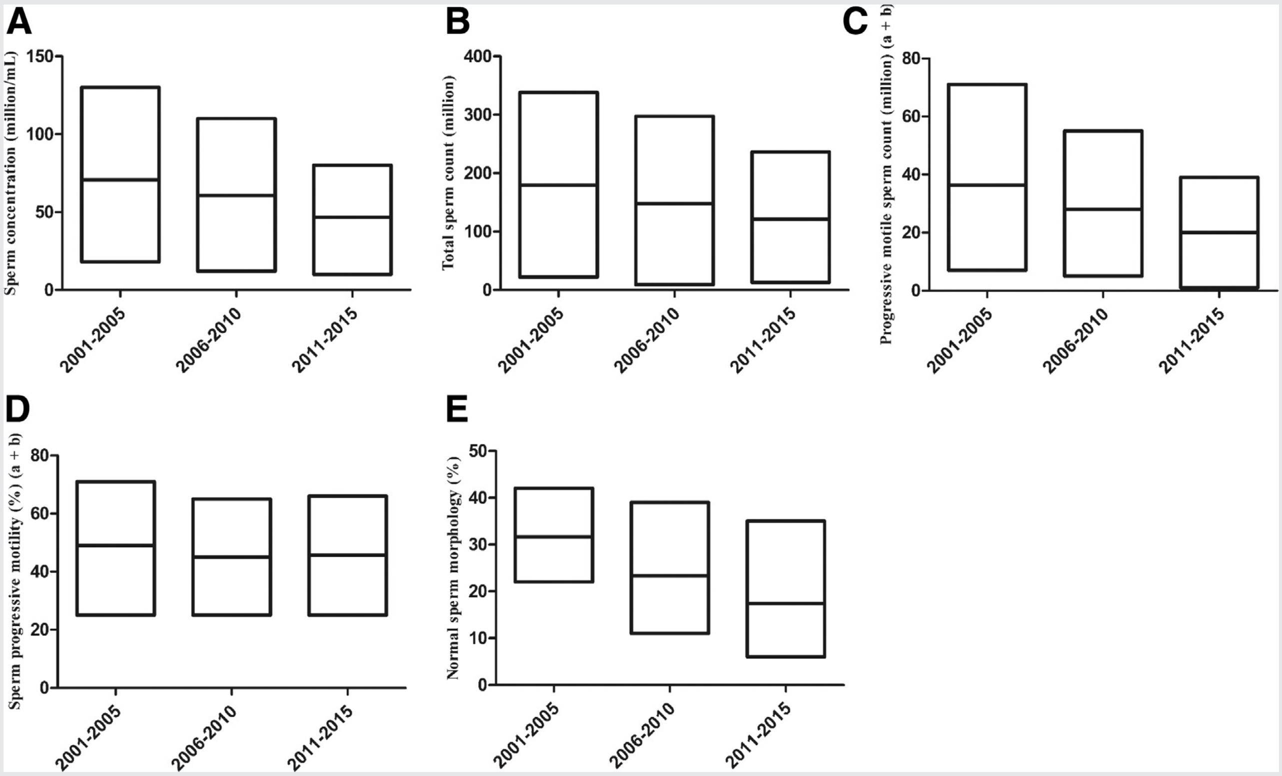
SUPPLEMENTAL FIGURE 1



Semen quality of 30,636 young men from the general population in Hunan, China. Semen parameters of Chinese young men from the general population. The bars show the 5th to 95th percentiles with median lines. (**A**) Sperm concentration, (**B**) total sperm count, (**C**) progressive motile sperm count, (**D**) sperm progressive motility, and (**E**) normal sperm morphology decreased during the 15-year period.

Huang. Declining semen quality in Chinese men. Fertil Steril 2016.

Five Studies Proving Glyphosate Herbicide Causes Parkinson disease
五项研究证实草甘膦除草剂造成帕金森疾病

附录 A：草甘膦造成帕金森病铁证 1
Study A:

Levodopa/benserazide 500/125 mg daily provided satisfactory clinical outcome.

Mov Disord. 2001 May;16(3):565-8.
运动失调杂志，2001 年 5 月；16(3):565-8.

Parkinsonism after glycine-derivate exposure.
氨基乙酸派生物接触后造成帕金森病

作者：Barbosa ER, Leiros da Costa MD, Bacheschi LA, Scaff M, Leite CC.

Source
作者单位：
Divisão de Clínica Neurológica, Hospital das Clínicas da Faculdade, Medicina da Universidade, São Paulo, São Paulo, Brazil. egbertob@8415.com.br
巴西医学大学临床医院神经学部

Abstract
摘要
This 54-year-old man accidentally sprayed himself with the chemical agent glyphosate, a herbicide derived from the amino acid glycine. He developed disseminated skin lesions 6 hours after the accident. One month later, he developed a symmetrical parkinsonian syndrome. Two years after the initial exposure to glyphosate, magnetic resonance imaging revealed hyperintense signal in the globus pallidus and substantia nigra, bilaterally, on T2-weighted images. Levodopa/benserazide 500/125 mg daily provided satisfactory clinical outcome.
这位 54 岁男性无意中对自己喷洒了草甘膦，氨基乙酸派生的除草剂。事故 6 小时后，他发生了弥散性皮肤损伤。一个月后，他发展了对称的帕金森病综合症。初次接触草甘膦后两年，磁共振成像，揭示 苍白球和塞美林氏神经节双边出现了 T2 加权像高信号。每天服用 500/125 mg 左旋多巴/苄丝肼获得了令人满意的临床效果。

链接：http://www.ncbi.nlm.nih.gov/pubmed/11391760

附录 B：草甘膦造成帕金森病铁证 2

Study B:

Arq Neuropsiquiatr. 2003 Jun;61(2B):381-6. Epub 2003 Jul 28. http://www.ncbi.nlm.nih.gov/pubmed/12894271

[Neuroimaging abnormalities in parkinsonism: study of five cases].

[Article in Portuguese]

da Costa Mdo D, Gonçalves LR, Barbosa ER, Bacheschi LA.

Clínica Neurológica do Hospital das Clínicas da Faculdade de Medicina da Univesidade de São Paulo, São Paulo, SP, Brasil.

Abstract

We report the brain magnetic resonance (MR) imaging abnormalities observed at the basal ganglia system of 5 patients (2 female and 3 male), who fulfilled the criteria of parkinsonism. The onset of parkinsonian syndrome ranged from 5 to 52 years old. All patients underwent MR exams with a 1.5T MR equipment. High field T2-weighted sequences disclosed hypersignal bilateral and symmetrically located exclusively at substantia nigra (3 cases), exclusively at globus pallidus (1case) and simultaneously at substantia nigra, globus pallidus and nigro-strital interconnections (1case). For three patients, the diagnose of secondary parkinsonism was supported by clinical data: the first had the onset of the symptoms after the exposure to an herbicide (glyphosate); the second after vaccination against measles; the third after coma due to encephalitis. For the other two patients, the onset of PS was progressive, resembling a typical idiopathic Parkinson's disease (PD) but the findings at the MR dimissed this initial diagnose. In this study, the contribution of neuroimaging was crucial to recognize secondary parkinsonism though the ethiological agents could not be determined in these patients.

Arq Neuropsiquiatr. 2003 Jun;61(2B):381-6. Epub 2003 Jul 28.

神经精神病学档案。2003 年 6 月；61(2B):381-6.上线发表日期：2003 年 7 月 28 日

[Neuroimaging abnormalities in parkinsonism: study of five cases].

帕金森病中的神经影像学异常：五项案例的研究

[Article in Portuguese]

[原文葡萄牙文]

作者：da Costa Mdo D, Gonçalves LR, Barbosa ER, Bacheschi LA.

Source

作者单位：

Clínica Neurológica do Hospital das Clínicas da Faculdade de Medicina da Univesidade de São Paulo, São Paulo, SP, Brasil.

巴西医学大学临床医院神经学部

Abstract

摘要

We report the brain magnetic resonance (MR) imaging abnormalities observed at the basal ganglia system of 5 patients (2 female and 3 male), who fulfilled the criteria of parkinsonism. The onset of parkinsonian syndrome ranged from 5 to 52 years old. All patients underwent MR exams with a 1.5T MR equipment. High field T2-weighted sequences disclosed hypersignal bilateral and symmetrically located exclusively at substantia nigra (3 cases), exclusively at globus pallidus (1case) and simultaneously at substantia nigra, globus pallidus and nigro-strital interconnections (1case). For three patients, the diagnose of secondary parkinsonism was supported by clinical data: the first had the onset of the symptoms after the exposure to an herbicide (glyphosate); the second after vaccination against measles; the third after coma due to encephalitis. For the other two patients, the

onset of PS was progressive, resembling a typical idiopathic Parkinson's disease (PD) but the findings at the MR dimissed this initial diagnose. In this study, the contribution of neuroimaging was crucial to recognize secondary parkinsonism though the ethiological agents could not be determined in these patients.

我们报告对 5 位患者（两女三男）在基底节观察到的脑磁共振图像异常，他们都满足了帕金森病的标准。… 第一位患者在接触除草剂（草甘膦）后出现了帕金森病的症状。

信息来源：http://www.ncbi.nlm.nih.gov/pubmed/12894271

附录 C：草甘膦造成帕金森病铁证 3

Study C:

Am J Epidemiol. 2007 Feb 15;165(4):364-74. Epub 2006 Nov 20. http://www.ncbi.nlm.nih.gov/pubmed/17116648

Pesticide exposure and self-reported Parkinson's disease in the agricultural health study.

Kamel F, Tanner C, Umbach D, Hoppin J, Alavanja M, Blair A, Comyns K, Goldman S, Korell M, Langston J, Ross G, Sandler D.
National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA. kamel@mail.nih.gov

Abstract
Previous studies based on limited exposure assessment have suggested that Parkinson's disease (PD) is associated with pesticide exposure. The authors used data obtained from licensed private pesticide applicators and spouses participating in the Agricultural Health Study to evaluate the relation of self-reported PD to pesticide exposure. Cohort members, who were enrolled in 1993-1997, provided detailed information on lifetime pesticide use. At follow-up in 1999-2003, 68% of the cohort was interviewed. Cases were defined as participants who reported physician-diagnosed PD at enrollment (prevalent cases, n = 83) or follow-up (incident cases, n = 78). Cases were compared with cohort members who did not report PD (n = 79,557 at enrollment and n = 55,931 at follow-up). Incident PD was associated with cumulative days of pesticide use at enrollment (for highest quartile vs. lowest, odds ratio (OR) = 2.3, 95% confidence interval: 1.2, 4.5; p-trend = 0.009), with personally applying pesticides more than half the time (OR = 1.9, 95% confidence interval: 0.7, 4.7), and with some specific pesticides (ORs > or = 1.4). Prevalent PD was not associated with overall pesticide use. This study suggests that exposure to certain pesticides may increase PD risk. Findings for specific chemicals may provide fruitful leads for further investigation.

Am J Epidemiol. 2007 Feb 15;165(4):364-74. Epub 2006 Nov 20.
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Pesticide exposure and self-reported Parkinson's disease in the agricultural health study.

农业健康研究中农药接触与自己报告的帕金森病

Authors/作者：Kamel F, Tanner C, Umbach D, Hoppin J, Alavanja M, Blair A, Comyns K, Goldman S, Korell M, Langston J, Ross G, Sandler D.

Source
作者单位：
National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709, USA.
kamel@mail.nih.gov
美国国家环境健康科学研究院

Abstract

摘要

Previous studies based on limited exposure assessment have suggested that Parkinson's disease (PD) is associated with pesticide exposure. The authors used data obtained from licensed private pesticide applicators and spouses participating in the Agricultural Health Study to evaluate the relation of self-reported PD to pesticide exposure. Cohort members, who were enrolled in 1993-1997, provided detailed information on lifetime pesticide use. At follow-up in 1999-2003, 68% of the cohort was interviewed. Cases were defined as participants who reported physician-diagnosed PD at enrollment (prevalent cases, n = 83) or follow-up (incident cases, n = 78). Cases were compared with cohort members who did not report PD (n = 79,557 at enrollment and n = 55,931 at follow-up). Incident PD was associated with cumulative days of pesticide use at enrollment (for highest quartile vs. lowest, odds ratio (OR) = 2.3, 95% confidence interval: 1.2, 4.5; p-trend = 0.009), with personally applying pesticides more than half the time (OR = 1.9, 95% confidence interval: 0.7, 4.7), and with some specific pesticides (ORs > or = 1.4). Prevalent PD was not associated with overall pesticide use. This study suggests that exposure to certain pesticides may increase PD risk. Findings for specific chemicals may provide fruitful leads for further investigation.

基于有限接触评估的以前研究提议帕金森病与农药接触关联。作者们使用了自《农业健康研究》中有证书的私人农药使用者及其配偶的数据来评价自己报告的帕金森病与农药接触之间的关系。… 该研究提议某些农药可能提高帕金森病的风险。对具体化学品的发现对进一步研究提供有成效的线索。

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全文链接：http://aje.oxfordjournals.org/content/165/4/364.full.pdf

全文披露：接触农药后来患帕金森病中接触草甘膦者比例较高，表明与草甘膦关联性较高。

附录 D：草甘膦造成帕金森病铁证 4

Study D:

Parkinsonism & Related Disorders http://www.prd-journal.com/article/S1353-8020(11)00041-1/abstract
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Parkinsonism after chronic occupational exposure to glyphosate☆

Gang Wang, Xiao-Ning Fan, Yu-Yan Tan, Qi Cheng, Sheng-Di Chen

Department of Neurology & Institute of Neurology, Ruijin Hospital affiliated to Shanghai Jiaotong University School of Medicine, Shanghai 200025, China

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Parkinsonism after chronic occupational exposure to glyphosate.

慢性职业性接触草甘膦造成帕金森病（震颤麻痹综合症）

Parkinsonism Relat Disord. 2011 Jul;17(6):486-7.
发表刊物：《帕金森病相关失调》，2011 年 7 月；17(6):486-7.

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doi: 10.1016/j.parkreldis.2011.02.003，上线发表日期 2011 年 3 月 2 日

Authors: Wang G, Fan XN, Tan YY, Cheng Q, Chen SD.
作者：Gang Wang（王刚）、Xiao-ning Fan（范小宁）、Tan YY（谭 YY）、Cheng Q（程 Q）、Sheng-di Chen（陈生弟）
摘要：http://www.ncbi.nlm.nih.gov/pubmed/21367645?report=abstract
更多内容：
http://www.deepdyve.com/lp/elsevier/parkinsonism-after-chronic-occupational-exposure-to-glyphosate-wpE2EMQQ6q

As a broad-spectrum herbicide employed to kill weeds, glyphosate (N-(phosphonomethyl) glycine) is typically either sprayed to be absorbed through the leaves, injected into the trunk, or applied to the stump of a tree, and is also used to control vegetation around transmission towers, pipelines, water drainage channels, public squares, and streets throughout the world.
作为一种光谱除草剂，草甘膦在世界各地用来杀死野草，通常或者喷洒到叶子上以便吸收，注射到树干中，或者应用于树墩，还用来治理输电塔、管道、排水渠、公共广场与道路旁的植物。

In China, glyphosate is popularly used as a hypotoxic weed-killer in rural areas. Every year, there are reports of acute intoxication of glyphosate due to attempted suicide or error in usage among adults and children. [2] Symptoms in such cases are frequently reported as consisting of digestive tract dysfunction, circulatory and respiratory failure, and liver and kidney damage [1, 2].
在中国，草甘膦在农村作为一种弱毒性除草剂普遍使用。每年都有成年人与儿童故意自杀或者误服草甘膦造成急性中毒的报告。[2] 这样的事例中经常报告的症状包括消化系统失调、血液循环与呼吸系统衰竭，以及肝脏和肾脏损伤。[1,2]

Neurological involvement, in particular extrapyramidal symptoms and signs including limb rigidity and resting tremor has only been reported following a few isolated events rather than in the setting of chronic occupational exposure [3].
神经系统的参与，尤其是锥体外系症状与肢体刚直与静止性震颤，仅在个别孤立事件续后有所报告，但是一直没有慢性职业性接触背景下的这种报告[3]。

Here we report a patient with parkinsonism following chronic occupational exposure to glyphosate. A previously healthy 44-year-old woman presented with rigidity, slowness and resting tremor in all four limbs with no impairment of short-term memory, after sustaining long term chemical exposure to glyphosate for 3 years as a worker in a chemical factory. The chemical plant produced a range of herbicides including: glyphosate, gibberellins, and dimethyl hydrogen phosphate; however, the patient worked exclusively in the glyphosate production division. She only wore basic protection such as gloves or a face mask for 50 h each week in the plant where glyphosate vapor was generated. She frequently felt weak. Two months before she came to our clinic, she had experienced severe dizziness and blurred vision.

这里我们报告一个慢性职业性接触草甘膦续后患帕金森病的患者情况。她 44 岁，原先身体健康，在一个化工厂作为公认持续三年接触草甘膦后，四肢存在刚直、慢性与静止性震颤，但是短期记忆没有损伤。这家化工厂生产一系列除草剂，包括草甘膦、赤霉素，与二甲基磷酸氢盐；然而，这位患者仅在草甘膦生产部门工作。工作期间她仅戴手套或口罩这样的基本保护，在产生草甘膦气体的部门每周工作 50 小时。她经常感到虚弱。来我们医院前两个月，她感到严重头昏眼花、视觉模糊。

After being diagnosed by the local doctor with cervical spondylosis, the patient received treatment with DAN-SHEN (salvia) injections for one week without any improvement.

当地医生诊断为颈椎病以后，给患者注射了一周丹参（鼠尾草），但是没有任何改善。

Physical examination revealed a parkinsonian syndrome. There was no known family history of neurological or other relevant disorders. The patient had consumed no other medications or herbal preparations before the onset of symptoms.

身体检查揭示了帕金森病综合症。患者没有任何已知的神经学的或相关失调家族史。帕金森病症状开始前，患者没有获得任何其他医药治疗或者草药治疗。

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No report of parkinsonism induced by glyphosate after occupational exposure has been published to date.

到目前为止，没有公开发表过任何慢性职业性接触草甘膦诱发帕金森病的任何报告。

In 2001, Barbusa et al. reported a case resulting from spraying glyphosate in a garden without wearing protection [3]. The patient had acute skin lesions one week after the chemical exposure and displayed rigidity and slowness in all four limbs one month after the initial exposure. One year later, he developed a slow resting tremor in the left hand and arm, accompanied by impairment of short-term memory.

2001 年，Barbusa et al.报告过在花园中喷洒草甘膦而没有戴任何防护面具的一个案例 [3]。化学接触一周后，患者发生急性皮肤损害，而且，在初次接触草甘膦后一个月四肢显示刚直性与缓慢性。一年后，患者左手与左臂发展了慢性静止性震颤，同时出现短期记忆损伤。

Although our patient had similar extrapyramidal symptoms, she had neither skin lesions nor memory loss.
我们的患者，尽管存在类似的锥体外系症状，她既没有皮肤损伤，也没有记忆损伤。

In a series of experiments, glyphosate demonstrated a wide range of toxicities for enzymes such as cholinesterase, carboxylesterase, and glutathione S-transferase [4].
一系列的实验中，草甘膦显示对胆碱酯酶、羧酸酯酶，与谷胱苷肽 S-转移酶这些酶造成广泛一系列毒性[4]。

Recently, a case of glyphosae-surfactant induced reversible encephalopathy was reported and it was suggested that glyphosate-surfactant could induce a prolonged but reversible encephalopathy suggestive of acute central nervous system toxicity different from previously reported symptoms and disorder including: nausea, vomiting, oral and abdominal pain, renal and hepatic impairment, and pulmonary edema [5].
不久前，报告了一项草甘膦—表面活性剂诱发的可逆性脑病案例，它提议草甘膦—表面活性剂能够诱发延长的但是可逆性脑部，提议存在着一种急性中枢神经毒性，其症状与以前报告的症状与失调有所不同：恶心、呕吐、口腔与腹部疼痛、肾脏与肝脏损伤，以及肺水肿[5]。

In these previous investigations [1, 3, 5], the neurotoxicity of glyphosate was suggested to be via an excitotoxic mechanism. Unfortunately, there are to date no confirmed studies of neurotoxicity focused on the relationship between dopaminergic neuro transmission and glyphosate in the literature.
以前的这些调查中 [1, 3, 5]，草甘膦的神经毒性被建议为通过一种兴奋性毒性机制。遗憾的是，到目前为止，科学文献中还没有聚焦于多巴胺能神经传递与草甘膦之间关系的确认研究。

附录 E：草甘膦造成帕金森病铁证 5

Study E:

http://www.ncbi.nlm.nih.gov/pubmed/22504123

Neurotoxicol Teratol. 2012 May-Jun;34(3):344-9. doi: 10.1016/j.ntt.2012.03.005. Epub 2012 Apr 4.

Glyphosate induced cell death through apoptotic and autophagic mechanisms.

Gui YX, Fan XN, Wang HM, Wang G, Chen SD.

Department of Neurology & Institute of Neurology, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China.

Abstract

Herbicides have been recognized as the main environmental factor associated with human neurodegenerative disorders such as Parkinson's disease(PD). Previous studies indicated that the exposure to glyphosate, a widely used herbicide, is possibly linked to Parkinsonism, however the underlying mechanism remains unclear. We investigated the neurotoxic effects of glyphosate in differentiated PC12 cells and discovered that it inhibited viability of differentiated PC12 cells in dose-and time-dependent manners. Furthermore, the results showed that glyphosate induced cell death via autophagy pathways in addition to activating apoptotic pathways. Interestingly, deactivation of Beclin-1 gene attenuated both apoptosis and autophagy in glyphosate treated differentiated PC12 cells, suggesting that Beclin-1 gene is involved in the crosstalk between the two mechanisms.

Glyphosate induced cell death through apoptotic and authophagic mechanisms

草甘膦通过 凋亡 和 authophagic 机制诱导细胞死亡

Neurotoxicology and Teratology, Volume 34, Issue 3, May–June 2012, Pages 344–349

《神经病理学与畸形学》杂志，34 卷第 3 期，2012 年 5-6 月，344-349 页

http://www.sciencedirect.com/science/article/pii/S0892036212000438

Authors/作者：Ya-xing Gui[a,b], Xiao-ning Fan[a, b], （范小宁）, Hong-mei Wang[a], （王红梅）, Gang Wang[a], （王刚，通讯作者）, Sheng-di Chen[a, b], （陈生弟，通讯作者，）

[a] Department of Neurology & Institute of Neurology, Ruijin Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

上海交通大学医学院附属上海瑞金医院神经病学科与神经病学研究所，

[b] Lab of Neurodegenerative Diseases & key Laboratory of Stem Cell Biology, Institute of Health Science, Shanghai Institutes of Biological Sciences, Chinese Academy of Science & Shanghai Jiao Tong University School of Medicine, Shanghai 200025, China

上海科学院上海生物科学研究所、健康科学研究所、肝细胞生物学神经组织退化疾病与重点实验室，与上海交通大学医学院

Abstract

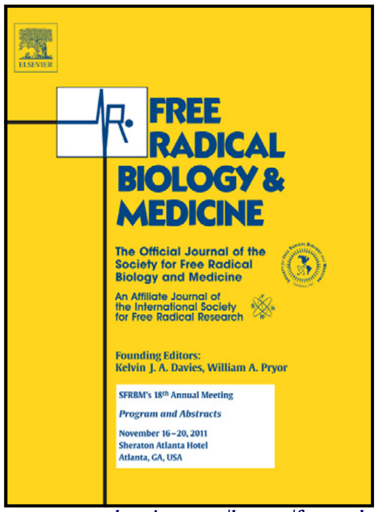
摘要

Herbicides have been recognized as the main environmental factor associated with human neurodegenerative disorders such as Parkinson's disease(PD). Previous studies indicated that the exposure to glyphosate, a widely used herbicide, is possibly linked to Parkinsonism, however the underlying mechanism remains unclear. We investigated the neurotoxic effects of glyphosate in differentiated PC12 cells and discovered that it inhibited viability of differentiated PC12 cells in dose-and time-dependent manners. Furthermore, the results showed that glyphosate induced cell death via autophagy pathways in

addition to activating apoptotic pathways. Interestingly, deactivation of Beclin-1 gene attenuated both apoptosis and autophagy in glyphosate treated differentiated PC12 cells, suggesting that Beclin-1 gene is involved in the crosstalk between the two mechanisms.

除草剂被承认是与人类帕金森病这样的神经组织退化性失调相关的主要环境因素。以前的研究表明，接触于广泛使用的草甘膦除草剂，可能与帕金森病（震颤性麻痹症）关联，然而，作为其基础的机制依然不清楚。我们在分化型 PC12 细胞中研究了草甘膦的毒害神经作用，发现草甘膦以剂量—时间依赖性方式抑制分化型 PC12 细胞的发育能力。此外，研究的结果表明，在激活凋亡通路之外，草甘膦还通过细胞自我吞噬作用同路诱发细胞的死亡。有意思的是，在草甘膦处理的分化型 PC12 细胞，钝化 Beclin-1 基因减弱细胞凋亡及其自我吞食作用，提议 Beclin-1 基因涉入了这两个机制之间的串扰。

Author's Accepted Manuscript



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Roundup Disrupted Male Reproductive Functions By Triggering Calcium-Mediated Cell Death In Rat Testis And Sertoli Cells

Vera Lúcia de Liz Oliveira Cavalli, Daiane Cattani, Carla Elise Heinz Rieg, Paula Pierozan, Leila Zanatta, Eduardo Benedetti Parisotto, Danilo Wilhelm Filho, Fátima Regina Mena Barreto Silva, Regina Pessoa-Pureur, Ariane Zamoner

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ROUNDUP DISRUPTED MALE REPRODUCTIVE FUNCTIONS BY TRIGGERING CALCIUM-MEDIATED CELL DEATH IN RAT TESTIS AND SERTOLI CELLS

Vera Lúcia de Liz Oliveira Cavalli[1], Daiane Cattani[1], Carla Elise Heinz Rieg[1], Paula Pierozan[2], Leila Zanatta[1], Eduardo Benedetti Parisotto[3], Danilo Wilhelm Filho[3], Fátima Regina Mena Barreto Silva[1], Regina Pessoa-Pureur[2], Ariane Zamoner[1*]

[1]Departamento de Bioquímica, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis - Santa Catarina, Brazil

[2]Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre – Rio Grande do Sul, Brazil

[3]Departamento de Ecologia e Zoologia, Centro de Ciências Biológicas, Universidade Federal de Santa Catarina, Florianópolis - Santa Catarina, Brazil

*Corresponding author: Dr. Ariane Zamoner. Departamento de Bioquímica, Centro de Ciências Biológicas, UFSC. Campus Universitário, Bairro Trindade, CEP: 88040-970; Florianópolis, Santa Catarina, Brazil. e-mail: ariane.zamoner@ufsc.br, Tel/Fax: +55 48 3721 4747 / + 55 48 3721 9672

Abstract

Glyphosate is the primary active constituent of the commercial pesticide Roundup®. The present results show that acute Roundup® exposure at low doses (36 ppm, 0.036 g/L) for 30 min induces oxidative stress and activates multiple stress-response pathways leading to Sertoli cell death in prepubertal rat testis. The pesticide increased intracellular Ca^{2+} concentration by opening L-type voltage-dependent Ca^{2+} channels (L-VDCC) as well as endoplasmic reticulum IP_3 and ryanodine receptors, leading to Ca^{2+} overload within the cells, which set off oxidative stress and necrotic cell death. Similarly, 30 min incubation of testis with glyphosate alone (36 ppm) also increased $^{45}Ca^{2+}$ uptake. These events have been prevented by the antioxidants Trolox® and ascorbic acid. Activated protein kinase C (PKC), phosphatidylinositol-3-kinase (PI3K) and the mitogen-activated protein kinases (MAPKs), such as ERK1/2 and p38MAPK have played a role in eliciting Ca^{2+} influx and cell death. Roundup® decreased the levels of reduced glutathione (GSH) and increased the amounts of thiobarbituric reactive species (TBARS) and protein carbonyls. Also, exposure to the glyphosate-Roundup® has stimulated the activity of glutathione peroxidase, glutathione reductase, glutathione-S-transferase, gamma-glutamyl transferase (γGT), catalase, superoxide dismutase and glucose-6-phosphate dehydrogenase, supporting downregulated GSH levels. Glyphosate has been described as an endocrine disruptor affecting the male reproductive system; however, the molecular basis of its toxicity remains to be clarified. We could propose that Roundup® toxicity, implicating in Ca^{2+} overload, cell signaling misregulation, stress response of the endoplasmic reticulum and/or depleted antioxidant defenses could contribute to Sertoli cell disruption of spermatogenesis that could impact male fertility.

Key words: glyphosate-Roundup®; cell signaling; Sertoli cell; oxidative stress; calcium homeostasis; cell death.

Introduction

Brazil is the world largest consumer of pesticides. This leadership might lead to a large number of health problems for occupationally exposed workers, their families and the environment [1]. Glyphosate [N-(phosphonomethyl)glycine] formulations are the widely used herbicides in agriculture worldwide. Although glyphosate is described as the primary active ingredient present in Roundup® (Monsanto Company, St, Louis, MO), this commercial formulation has greater side effects than glyphosate alone [2-3]. Moreover, it has been demonstrated that toxic events triggered by Roundup® might be due to the synergistic effects between glyphosate and other formulation products [4-6]. The adjuvants are considered as inert, however, the polyethoxylated tallowamine (POEA) is toxic and could facilitate glyphosate penetration through plasmatic membranes and consequently potentiate its action and toxicity [3,7-10]. In this context, Mesnage *et al*. [11] demonstrated POEA as one of the active ingredients of Roundup® formulations inducing human toxicity, triggering necrosis by disrupting cell membranes around its critical micellar concentration, rather than glyphosate that leads to apoptosis. Moreover, previous studies have supported the possibility that mixtures of glyphosate and surfactant can aggravate cellular damage, inducing cell death [4-6,8,9,12-17]. Also, the surfactant nonylphenol, an important environment contaminant, alters Ca^{2+} homeostasis and leads to Sertoli cell apoptosis [18]. Glyphosate alone or Roundup®, were found to induce significant changes in cellular antioxidant status leading to glutathione depletion, enzymatic disorders, and increased lipid peroxidation in keratinocytes [14,19].

The cytotoxicity provoked by several toxic agents is associated with loss of intracellular Ca^{2+} homeostasis. The imbalance in Ca^{2+} physiology is believed to be associated with misregulation of Ca^{2+} intracellular stores and/or increased permeability of the biomembranes to this ion. Intracellular Ca^{2+} overload can underlie mitochondrial dysfunctions which involve several molecular events, including activation of signaling pathways, in addition to reactive oxygen species (ROS) overproduction that could culminate in cell death [17,20-22].

Nowadays, an important challenge concerning the deleterious effects of pesticides in occupational exposed agricultural workers is the high prevalence of reproductive dysfunctions observed in this population [23-26]. Glyphosate is supposed to be specific on plant metabolism; however, side effects in animals and humans have been claimed. In this context, glyphosate might act as an endocrine disruptor affecting the male reproductive system, since it can lead to alterations on aromatase activity and expression [8], estrogen-regulated genes [27] and testosterone levels [10,16]. Moreover, Roundup®, the commercial formulation of glyphosate, disrupts spermatogenesis and causes loss of fertility, reinforcing its toxicity to testicular cells. Also, in MA-10 Leydig tumor cell line, Roundup® inhibits the steroidogenesis by disrupting the expression of the StAR proteins [12]. In addition, Dallegrave and colleagues [28] have demonstrated that glyphosate-Roundup exposure during pregnancy and lactation did not induce maternal toxicity in Wistar rats, but, induced adverse reproductive effects on male offspring rats including decreased daily sperm production during adulthood, increased percentage of abnormal sperms and decreased testosterone serum level at puberty. Conversely, the authors observe only a vaginal canal-opening delay in exposed female offspring. Taken together, these data strongly suggest Roundup® as an endocrine disruptor affecting mainly male reproduction. However, the precise mechanisms underlying the effects of this pesticide on male reproductive tissue remains unclear. Although long term toxicity of Roundup® to animal tissues has been largely described [29], acute exposure to this pesticide is claimed to be toxic to fish [30]. Nevertheless, little information is available on the acute toxicity of low doses of Roundup® to mammal tissues, especially to the reproductive human male system.

ROS generation might be due to either physiological or pathological conditions. Enzymatic and non-enzymatic antioxidants are essential to maintain the redox status and serve as a defense against ROS [31]. In this context, when present at high levels, ROS play an essential role in the pathogenesis of many reproductive processes, considering their potential toxic effects to sperm quality and function. In addition, excessive ROS generation may induce DNA damage, accelerating germ cell death and causing decreased sperm counts. Altogether, these events could be associated with male infertility [32-34]. Environmental contaminants are known to modulate the antioxidant defense system and to cause oxidative stress in different species and cell types [6,19,35,36]. Our research group has previously demonstrated that the activity of antioxidant enzymes (superoxide dismutase - SOD, catalase -CAT, glutathione peroxidase - GPx, glutathione reductase - GR, and glutathione-S-transferase - GST) as well as the reduced glutathione (GSH) levels, could be affected by endocrine diseases, such as hypo- and hyperthyroidism, leading to oxidative stress in immature rat testis [37], thereby showing the participation of endocrine system in the redox potential of Sertoli cells. However, the effect of Roundup® in oxidative stress and antioxidant defenses in the testis remains to be clarified.

Therefore, we selected acute exposure of immature rat testis to low doses of this pesticide as a model of toxicity to the male reproductive system. In this study we investigated the molecular basis of the toxicity of this xenobiotic, focusing on the role of Ca^{2+} homeostasis, misregulation of signaling pathways and oxidative damage in the whole rat testis and in Sertoli cells in culture.

Materials and methods

Chemicals

Nifedipine, 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester) (Bapta-AM), N-[2-(p-Bromocinnamylamino) ethyl]-5-isoquinolinesulfonamide (H89), (Bisindoylmaleimidine IX, 2-{1-[3-(Amidinothio)propyl]-1H-indol-3-yl}-3-(1-methylindol-3-yl)maleimide ethanesulfonate salt) Ro 31-8220, Trolox [(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid], L-ascorbic acid, flunarizine, dantrolene sodium salt, Dulbecco's modified Eagle's medium (DMEM), Ham's F12 medium, penicillin, streptomycin, kanamycin and amphotericin B, Serum Replacement 3, bovine pancreas deoxyribonuclease (DNase type I), hyaluronidase (type I-S), trypsin, soybean trypsin inhibitor, sodium pyruvate, D-glucose, Hepes, and sodium bicarbonate were purchased from Sigma Chemical Company (St. Louis, MO, USA). Collagenase-Dispase and bovine serum albumin (BSA) were from Roche Diagnostics (Indianapolis, IN). $[^{45}Ca]CaCl_2$ (sp. act. 321 KBq/mg Ca^{2+}), and Optiphase Hisafe III biodegradable liquid scintillation were purchased from PerkinElmer (Boston, USA). Anti-p44/42 MAP Kinase (anti-ERK1/2), anti-phospho-p44/42 MAP kinase (anti-phospho ERK1/2), anti $p38^{MAPK}$ and anti-phospho $p38^{MAPK}$ antibodies were from Cell Signaling Technology, Inc. (USA). The herbicide Roundup Original® (Homologation number 00898793) containing glyphosate 360 g/L is a commercial formulation registered in the Brazilian Ministry of Agriculture, Livestock and Supply (Ministério da Agricultura, Pecuária e Abastecimento – MAPA). The Immobilon™ Western chemiluminescent HRP substrate was obtained from Millipore. All other chemicals were of analytical grade.

Animals

Wistar rats were bred in animal house and maintained in an air-conditioned room (about 21 ^{o}C) with controlled lighting (12 h/12 h light/dark cycle). Pelleted food (Nuvital, Nuvilab CR1, Curitiba, PR, Brazil) and tap water were available *ad libitum*. All animals' procedures were carried out in accordance with ethical recommendations of the Brazilian Veterinary Medicine Council and the Brazilian College of Animal Experimentation (Protocol CEUA/PP00471).

Primary Sertoli cell culture

Some experiments were carried out in Sertoli cells from 30-day-old Wistar rats. Rats were killed by decapitation, testes were removed and decapsulated. Sertoli cells were obtained by sequential enzymatic digestion as previously described by Dorrington *et al*. [39]. Sertoli cells from 30-day-old rat testis were seeded at the concentration of 650,000 cells/cm^2, in 24 well culture plates (Falcon, Deutscher, Brummath, France) and cultured for 72 h in Ham's F12/DMEM (1:1) medium supplemented with serum replacement 3, 2.2 g/L sodium bicarbonate and antibiotics (50,000 IU/L penicillin, 50 mg/L streptomycin, 50 mg/L kanamycin), fungicide (0.25 mg/L amphotericin B), in a humidified atmosphere of 5% CO_2:95% air at 32°C. Three days after plating, residual germ cells were removed by a brief hypotonic treatment using 20 mM Tris-HCl (pH

7.2) [40,41]. Cells were washed with PBS and fresh medium Ham's F12/DMEM (1:1) was added. On day 5 after plating, cells were used to study the effect of Roundup® on $^{45}Ca^{2+}$ uptake and cell viability, as described below.

$^{45}Ca^{2+}$ uptake

Whole testis or Sertoli cells in culture from 30-day-old male rats were preincubated in Krebs Ringer-bicarbonate (KRb) buffer (122 mM NaCl; 3 mM KCl; 1.2 mM $MgSO_4$; 1.3 mM $CaCl_2$; 0.4 mM KH_2PO_4; 25 mM $NaHCO_3$) for 15 min in a Dubnoff metabolic incubator at 34 °C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). Further, the medium was changed by fresh KRb and the whole testis or Sertoli cells were preincubated again with or without channel blockers, antioxidants or kinase inhibitors during 15 min before the pesticide addition and maintained during all the incubation period. The following drugs were used: nifedipine (10 µM), flunarizine (1 µM), U73122 (30 µM), LY294002 (10 µM), PD98059 (30 µM), SB239063 (10 µM), BAPTA-AM (50 µM), dantrolene (50 µM), H89 (10 µM), Ro 31-8220 (20 µM), Trolox® or ascorbic acid. After that, the medium was changed by fresh KRb with 0.1 µCi/mL $^{45}Ca^{2+}$ and incubated during 30 min in the absence (control) or presence of glyphosate-Roundup® (treated groups) ranging from 0.72 to 360 ppm (corresponding to 0.00072 to 0.360 g/L, respectively). Extracellular $^{45}Ca^{2+}$ was thoroughly washed off in a washing solution containing 127.5 mM NaCl, 4.6 mM KCl, 1.2 mM $MgSO_4$, 10 mM HEPES, 11 mM glucose, 10 mM $LaCl_3$, pH 7.3 (30 min in washing solution). The presence of La^{3+} during the washing stage was found to be essential to prevent release of the intracellular $^{45}Ca^{2+}$ [42]. After washing, tissue slices or cell cultures were digested and homogenized with 0.5 M NaOH solution, 100 µL aliquots were placed in scintillation fluid and counted in a LKB rack beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA), and 5 µL aliquots were used for protein quantification as described by Lowry and colleagues [43].

Measurement of lactate dehydrogenase (LDH) activity

After incubation of the testes or Sertoli cells in the absence or presence of the – glyphosate-Roundup® at nominal concentrations ranging from 0.72 to 360 ppm for 30 min, the incubation medium was collected for determination of extracellular LDH activity by a spectrophotometric method. The estimation of LDH activity was carried out by measuring the oxidation of NADH and the results were expressed as U/L/mg of protein.

[^{14}C] MeAIB accumulation

For amino acid accumulation experiments, rat testis were pre-incubated in KRb buffer for 30 min in a Dubnoff metabolic incubator at 34 °C, pH 7.4 and gassed with O_2:CO_2 (95:5; v/v). The testes were then incubated in fresh KRb buffer for 60 min. [^{14}C] MeAIB (3.7 kBq/mL) was added to each sample during the incubation period [44]. Glyphosate-Roundup® at 36 ppm (0.036 g/L) was added to incubation medium in the last 30 min incubation. After incubation, the slices were lysed in NaOH 0.5 M, the protein concentration was determined [43], 25 µL aliquots of tissue and external medium were placed in scintillation fluid and counted in a Beckman beta liquid scintillation spectrometer (model LS 6500; Multi-Purpose Scintillation Counter-Beckman Coulter, Boston, USA) for radioactivity measurements. The results were expressed as the tissue/medium (T/M) ratio: cpm/mL tissue fluid per cpm/mL incubation medium.

Antioxidant enzyme assays

For enzymatic activity, testis from control or Roundup®-treated groups were preincubated for 15 min in KRb buffer followed by incubation with or without 36 ppm glyphosate-Roundup during 30 min. After incubation time, tissue was homogenized in cold 0.1 M Tris buffer, pH 8.5 (10% homogenate w/v) to determine gamma-glutamyl transferase (GGT) activity or in 0.2 M Tris buffer, pH 7.4 to quantify glucose-6-phosphate dehydrogenase (G6PD) activity. Sample aliquots were saved for total protein determinations [43].

In order to determine catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferase (GST) activities, after treatment with 36 ppm glyphosate-Roundup® for 30 min the testis were homogenized in a cold buffer containing: 20 mM sodium phosphate pH 7.4, 0.1% Triton and 150 mM NaCl (1:20 w/v). The determinations were performed using the supernatant after centrifugation of the homogenate (5,000 *g* for 5 min).

a. Gamma-glutamyl transferase assay

GGT activity was measured by using the method previously described by Orlowsky and Meister [45], using L-γ-glutamyl *p*-nitroanilide as substrate and glycylglycine as the acceptor molecule.

Aliquots of the tissue homogenate prepared as described above were incubated with the enzymatic substrate. The reaction was allowed to proceed for 60 min at 37 °C and the enzymatic reaction was stopped by addition of acetic acid. The absorbance of the samples was determined in a plate reader (Tecan Infinite® 200 PRO) at 530 nm. The results were expressed as U/L/μg protein**.**

b. Glucose-6-phosphate dehydrogenase assay

For measuring the G6PD activity, aliquots of tissue homogenate were incubated in the presence of $NADP^+$ leading to the oxidation of glucose-6-phosphate to 6-phosphogluconate. The NADPH produced was measured in a kinetic mode during 10 minutes. The results were calculated by assessing the increase of the optical density per minute (slope) of the sample against the "slope" of standard G6PD enzyme activity. The G6PD assay kit was kindly provided by Intercientífica (São José dos Campos, SP, Brazil).

c. Catalase activity

The CAT activity was determined in tissue homogenates according to the method described by Aebi [46], which is based on measuring the decreased absorbance in a 10 mM hydrogen peroxide solution at 240 nm for 30 seconds. The enzyme activity was expressed as $mmol.min^{-1}.g^{-1}$.

d. Superoxide dismutase activity

The SOD activity was analyzed according to the method described by Misra and Fridovich [47] and modified by Boveris *et al*. [48]. The reaction is based on the epinephrine oxidation (pH 2.0 to pH 10.2) which produces superoxide anion and adrenochrome, which is measured at 480 nm. A unit of SOD is defined as the amount of enzyme that inhibits the speed of oxidation of adrenalin by 50% and the result expressed in USOD g^{-1}.

e. Glutathione peroxidase activity

The GPx activity was analyzed by using the method described by Flohé and Günzler [49]. This method is based on the tert-butyl hydroperoxide (t-BuOOH) reduction via oxidation of GSH to GSSG, catalyzed by GPx, and subsequent regeneration of GSH by the enzyme GR with oxidation of NADPH at 340 nm. Therefore, the rate of oxidation of NADPH is proportional to the activity of the GPx in the sample. The enzyme activity was expressed as $\mu mol.min^{-1}.g^{-1}$.

f. Glutathione reductase activity

The GR activity was determined by the method of Carlberg and Mannervik [50], which measured the rate of NADPH oxidation at 340 nm due to the formation of GSH, from GSSG, by the action of GR present in the sample. The unit of enzyme activity was expressed as $\mu mol.min^{-1}.g^{-1}$.

g. Glutathione S-transferase activity

The GST activity was measured by using the methodology described by Habig *et al*. [51]. In this protocol, the 1-chlore-2,4-dinitrobenzene (CDNB, as substrate for GST) was used. In this reaction, GST promotes the CDNB–GSH conjugation. This reaction was spectrophotometrically monitored for 60 s at 340 nm. The enzyme activity was expressed as $\mu mol.min^{-1}.g^{-1}$.

Reduced glutathione assay

The reduced glutathione (GSH) was determined according to Beutler *et al*. [52], using the reagent DTNB (5,5'-dithiobis 2-nitrobenzoic acid). After being centrifuged at 5,000 *g* for 5 min, the supernatants from the acid extracts (TCA 12%, 1:10 w/v) were added to 2.5 mM DTNB in 0.2 M sodium phosphate buffer pH 8.0, and the formation of the thiolate anion of yellow color was immediately measured at 412 nm. Determinations were expressed in $\mu mol.g^{-1}$.

Quantification of lipoperoxidation levels

The endogenous lipid peroxidation was evaluated in the testes by detection of substances that react with thiobarbituric acid (TBARS), particularly malondialdehyde (MDA), according to the method described by Bird and Draper [53]. Briefly, the homogenate was precipitated with trichloroacetic acid (TCA 12%) followed by the incubation with buffer 60 mM Tris–HCl pH 7.4 (0.1mM DPTA) and TBA 0.73%, at 100 °C, for 60 min. After cooling, the samples were then centrifuged (5 min. at 10,000 *g*) and the absorbance of the chromophore measured at 535 nm. The values were expressed in nmol $TBARS.g^{-1}$.

Protein carbonyl assay

The oxidative damage to proteins by carbonylation was determined by the method described by Levine et al [54]. Soluble protein was reacted with 10 mM DNPH (2,4-dinitrophenyl-hydrazine) in 2M hydrochloric acid for 1 h at room temperature in the dark and precipitated with trichloroacetic acid (TCA 20%). After centrifugation (11,000 *g* for 5 min) the pellet protein was washed thrice by resuspension in ethanol/ethyl acetate (1:1). Proteins were then solubilized in 6M guanidine hydrochloride in 20 mM potassium phosphate and centrifuged at 14,000 *g* for 5 min to remove any trace of insoluble material. The carbonyl content was measured spectrophotometrically at 370 nm. The total proteins concentration was determined by the method of Lowry *et al*. [43] and the protein carbonyl concentration was expressed in μmol.mg^{-1} protein.

Western blot analysis

In some experiments testis were treated with or without 36 ppm Roundup during 30 min, then total tissue extracts were processed to Western blot analysis. Testis were homogenized in 300 μl of a lysis solution containing 2 mM EDTA, 50 mM Tris–HCl, pH 6.8, 4% (w/v) SDS and protein concentration was determined by the method of Lowry *et al.* (1951) using serum bovine albumin as the standard. Then, samples were dissolved in 25% (v/v) of a solution containing 40% glycerol, 5% mercaptoethanol, 50 mM Tris-HCl, pH 6.8 and boiled for 3 min. Equal protein concentrations were loaded onto 10 % polyacrylamide gels and analyzed by SDS-PAGE and transferred to nitrocellulose membranes for 1 h at 15 V in transfer buffer (48 mM Trizma, 39 mM glycine, 20% methanol and 0.25% SDS). The nitrocellulose membranes were washed for 10 min in Tris-buffered saline (TBS; 0.5 M NaCl, 20 mM Trizma, pH 7.5), followed by 2 h incubation in blocking solution (TBS plus 5% defatted dried milk). After incubation, the blot was washed twice for 5 min with TBS plus 0.05% Tween-20 (T-TBS), and then incubated overnight at 4 °C in blocking solution containing the following antibodies: anti-ERK1/2, anti-phospho ERK1/2, anti $p38^{MAPK}$, anti-phospho $p38^{MAPK}$ (diluted 1:2000). The blot was then washed twice for 5 min with T-TBS and incubated for 2 h in TBS containing peroxidase conjugated anti-rabbit IgG 1:2000. In addition, we used the following controls in the antibody experiments: primary antibody only, secondary antibody only and negative control (lacking the sample containing the antigen of interest). These controls stated the specificity and sensibility of the antibody. The blot was washed twice again for 5 min with T-TBS and twice for 5 min with TBS. The blot was then developed using a chemiluminescence ECL kit. Beta-actin immunocontent was used as protein loading. Western blots were quantified by scanning the films and determining optical densities with an OptiQuant version 02.00 software (Packard Instrument Company).

Statistical analysis

The results are means ± S.E.M.. When multiple comparisons were performed, evaluation was done using one-way ANOVA followed by Bonferroni multiple comparison test or Student *t* test. Differences were considered to be significant when $p < 0.05$.

Results

Involvement of Ca^{2+} on the mechanism of acute Roundup®-induced testis toxicity

Initially, rat testes were exposed to glyphosate-Roundup® at concentrations ranging from 0.72 to 360 ppm, corresponding to 0.00072 to 0.36 g/L, respectively, and $^{45}Ca^{2+}$ uptake was investigated. It is important to emphasize that Roundup is used in agricultural working at dilutions ranging from 10,000 ppm to 20,000 ppm (10 to 20 g/L), concentrations much higher than those described in our results. Results showed that Roundup® exposure for 30 min increased the $^{45}Ca^{2+}$ uptake at doses ranging from 7.2 to 36 ppm glyphosate; however, 360 ppm Roundup® lead to an important decrease in $^{45}Ca^{2+}$ influx (Figure 1A). In order to investigate whether the alterations in $^{45}Ca^{2+}$ uptake were related with cell death, the LDH release from the rat testis was measured. Interestingly, we observed an apparent link between $^{45}Ca^{2+}$ uptake and LDH release at 36 ppm (0.036 g/L) Roundup® (Figure 1B). Otherwise, at the higher Roundup® dose (360 ppm), the LDH release was higher, despite the decreased $^{45}Ca^{2+}$ uptake (Figure 1B). These findings strongly suggest that necrotic cell death could be directly related to Ca^{2+} toxicity up to 36 ppm of the pesticide. However, at very high concentrations (360 ppm), more complex mechanisms leading to necrotic cell death in the rat testis seem to be elicited by this xenobiotic. Therefore, in the present study, we were interested in investigating some mechanisms underlying Ca^{2+} toxicity in rat testis exposed to 36 ppm Roundup®.

Mechanisms of acute Roundup®-induced $^{45}Ca^{2+}$ uptake: Ca^{2+}channels and signaling pathways

The following experiments were carried out at 36 ppm Roundup®, a concentration able to induce the peaked $^{45}Ca^{2+}$ uptake and necrotic cell death. Figure 2A shows that Roundup®-induced $^{45}Ca^{2+}$ uptake occurred through L-type voltage-dependent Ca^{2+} channels (L-VDCCs), as demonstrated by using nifedipine (L-VDCC blocker). The role of the high intracellular Ca^{2+} levels in the Roundup®-triggered $^{45}Ca^{2+}$ uptake was evidenced by using BAPTA-AM (cell permeable Ca^{2+} quelator) and dantrolene (inhibitor of ryanodine receptors). Co-incubation of Roundup® and each drug was able to prevent the $^{45}Ca^{2+}$ uptake, suggesting the dependence on intracellular Ca^{2+} levels and Ca^{2+} releasing mediating the effects of the pesticide (Figure 2A). The use of dantrolene allowed us to set the implication of ryanodin receptors in the mechanism of action of Roundup®. In order to further clarify the mechanisms involved in Roundup®-mediated Ca^{2+} influx, the contribution of PLC, PKC and PKA to the pesticide effect was verified by using 30 μM U73122, 20 μM Ro-31-8220 and 10 μM H89 (PLC, PKC and PKA inhibitors, respectively). Results showed that U73122 was able to totally prevent the effect of Roundup® on Ca^{2+} uptake, while Ro 31-8220 partially prevented the Ca^{2+} influx, evidencing a PLC- and PKC-dependent mechanism, respectively. On the other hand, H89 was ineffective in preventing such effect, suggesting that PKA activation is not involved in the mode of action of this pesticide (Figure 2B).

The participation of other protein kinases such as PI3K, and MAPKs signaling pathways (MEK/ERK and p38MAPK) in the mechanism of action of 36 ppm Roundup® was investigated by using the specific inhibitors: LY294002 (10 μM), PD 98059 (30 μM) and SB239063 (10 μM), respectively. The outcomes showed that all the inhibitors used totally prevented the stimulatory effect of the pesticide on $^{45}Ca^{2+}$ uptake (Figure 2C), suggesting that the ability of Roundup® to increase $^{45}Ca^{2+}$ uptake could be associated to activation of several kinase pathways. Altogether, these findings demonstrate a role for PLC/PKC, PI3K, ERK and p38MAPK in Ca^{2+} influx induced by the pesticide in rat testis.

In order to verify whether the effects observed with Roundup® could be ascribed to glyphosate, the main active component of the pesticide, $^{45}Ca^{2+}$ uptake was measured in the presence of 36 ppm (0.036 g/L) glyphosate (in the absence of adjuvant/surfactant). Results showed increased $^{45}Ca^{2+}$ uptake induced by glyphosate. Moreover, the glyphosate-induced Ca^{2+} influx was partially prevented by nifedipine (Figure 2 D), suggesting a role for glyphosate in the toxicity of the pesticide.

Supporting the involvement of MAPK pathway in Roundup®-induced toxicity to testicular cells the Western blot analysis showed activated/phosphorylated ERK1/2 and p38MAPK in the prepubertal rat testis acutely exposed to pesticide (Figure 3).

Effect of antioxidants in Roundup®-induced $^{45}Ca^{2+}$ uptake and in cell viability

In order to verify the involvement of depleted non-enzymatic oxidative defenses on Roundup®-induced $^{45}Ca^{2+}$ uptake and necrotic cell death, the antioxidants ascorbic acid (vitamin C) and Trolox® (stable form of vitamin E) were used. Results showed that both antioxidants prevented the effect of the pesticide on $^{45}Ca^{2+}$ uptake, suggesting the contribution of oxidative events triggered by Roundup® in testicular cell toxicity (Figure 4A). Moreover, Trolox® totally prevented, while ascorbic acid only partially prevented the LDH release by Roundup® (Figure 4B). We, therefore, assessed other biochemical parameters to better evaluate the consequences of Roundup® exposure to the oxidative damage in rat testicular cells.

Effects of acute Roundup® exposure on biochemical parameters involved in oxidative damage

In order to attest the oxidative damage in the Roundup®-exposed testis, some biomarkers of oxidative damage were assessed. The content of thiobarbituric acid-reactive substances (TBARS), which is an indicator of lipid peroxidation, was significantly increased, as well as protein carbonyl levels, an indicator of oxidative damage to proteins, were found in the Roundup® groups when compared to controls (Figures 5A and 5B).

Once established the participation of oxidative events in the mechanism of toxicity of Roundup®, we sought to determine the enzymatic and non-enzymatic antioxidant defenses in Roundup®-treated rat testis. Results showed that exposure to the pesticide lead to decreased GSH levels. In addition, the activities of the enzymes involved in glutathione metabolism G6PD, GR, GPx, GST and γGT were significantly higher in Roundup®-treated rats than in controls. Roundup®-exposed rat testis also presented higher CAT and SOD activities (Figure 6) compared to controls.

Effect of Roundup® on neutral amino acid transport

Results showed that 36 ppm Roundup® lead to downregulation of the Na^+-coupled ^{14}C-α-methyl-amino-isobutyric acid (^{14}C-MeAIB) accumulation (Figure 7).

Effect of antioxidants in acute Roundup®-induced $^{45}Ca^{2+}$ uptake and LDH release in Sertoli cells

In order to investigate Sertoli cells as a target of Roundup® action within the testis, we examined the effects of the pesticide on $^{45}Ca^{2+}$ uptake and LDH release in primary cultures. Sertoli cell cultures from 30-day old rats were exposed to Roundup® at concentrations ranging from 0.72 to 360 ppm, and the influx of $^{45}Ca^{2+}$ was measured. Results showed that in Sertoli cells exposed to 36 ppm Roundup® for 30 min, both $^{45}Ca^{2+}$ influx and LDH release were increased (Figure 8A and B). Moreover, 360 ppm of the pesticide was able to increase $^{45}Ca^{2+}$ uptake and drastically decrease LDH release (Figure 8A and B). It is important to emphasize that according to those results obtained in whole testis, 36 ppm Roundup® was also able to peak $^{45}Ca^{2+}$ uptake and provoke necrotic cell death in cultured Sertoli cells. Moreover, the decreased Sertoli cell $^{45}Ca^{2+}$ uptake at 360 ppm Roundup® (Figure 8A) was also coincident with the most prominent LDH release (Figure 8B), as previously observed in whole testis (Figure 1). These results strongly suggest that Sertoli cells could be one of the main targets of Roundup® toxicity within rat testis.

To evaluate the role of depleted oxidative defenses, new experiments were carried out in cultured Sertoli cells supplemented with ascorbic acid and Trolox®. Interestingly, in agreement with the results obtained in whole testis, Trolox® (Figure 9A and 9B) and also ascorbic acid (Figure 9C and 9D) prevented Ca^{2+} overload and cell death in Sertoli cells in culture. We further assayed the effects of co-incubation of Trolox® and ascorbic acid in Roundup®-treated cells. The combination of both antioxidants used at physiological concentrations (75 μM ascorbic acid plus 50 μM

Trolox®) prevented both Ca^{2+} overload and necrotic cell death induced by Roundup®. On the other hand, 150 µM ascorbic acid plus 75 µM Trolox® were able to induce a *per se* effect on $^{45}Ca^{2+}$ uptake and necrotic cell death. Moreover, this effect was not modified by Roundup® exposure (Figure 9E and 9F).

Discussion

In the present study we shed light into the molecular mechanisms underlying the acute toxicity of Roundup® in the prepubertal male reproductive system. Acute Roundup® exposure to low doses induces L-VDCC-mediated Ca^{2+} influx and cell death. These events might be prevented, at least in part, by antioxidants. Activation of Ca^{2+} influx and necrotic cell death are dependent on PLC/PKC, PI3K and MAPK signaling pathways. PLC/IP_3 pathway together with ryanodine Ca^{2+} channels promote Ca^{2+} release from the endoplasmic reticulum, contributing to Ca^{2+} overloading. Activated enzymatic systems including SOD, CAT, GPx, GR, G6PD and GST support the decreased GSH levels found, while γGT affects GSH synthesis/turnover from extracellular amino acids. Depleted antioxidant defenses could underlie enhanced lipid and protein oxidation. The oxidative damage could misregulate cell function culminating in necrotic cell death (Figure 10).

It is important to note that the mechanisms of Roundup®-mediated toxicity in prepubertal rat testis were dependent on the concentration of the pesticide. In this context, Roundup® concentrations up to 36 ppm provoked Ca^{2+} overload, redox imbalance, disruption of cell signaling pathways and necrotic cell death in rat testis. Otherwise, at a ten-fold higher ppm-dose (360 ppm), the mechanisms underlying Roundup® toxicity seemed to be independent on Ca^{2+} influx. Our data demonstrate the complexity of the dose-dependent toxicity of this pesticide and suggest that apoptosis could not be a response to an acute insult with low doses of the pesticide. These results are in agreement with previous studies showing that the mechanisms of Roundup® toxicity changed around the critical micellar concentration of the surfactants [11].

Also, it is important to note that in the present study we demonstrate that glyphosate-Roundup® concentrations 10-folds more diluted than that recommended for herbicide action is highly toxic for humans. Our data contribute to evidence the high risk of handling this formulation, mainly in childhood and puberty, and the consequences for life.

The deleterious effects of Roundup® to the endocrine system of animals have been previously described by several researchers [12,16,28,55,56]. Also, the consequences of glyphosate exposure to testicular physiology became recognized from initial studies linking this herbicide with alterations in sperm quality, including decline in ejaculate volume, sperm concentration, semen initial fructose and semen osmolality [24]. In addition, a recent publication from Romano et al. [56] has demonstrated that glyphosate may disturb the masculinization process and promote behavioral changes, as well as histological and endocrine problems to male reproduction.

Our results showed that acute exposure to pure glyphosate, at the same concentration than Roundup® (36 ppm), was able to significantly enhance $^{45}Ca^{2+}$ uptake, and the mechanisms responsible for such effect seemed to involve L-VDCC.

Thus, we could propose a main role for glyphosate in Ca^{2+} overload toxicity of Roundup® in prepubertal rat testis; however, the possibility exists that POEA could alter or potentiate the cytotoxicity of the glyphosate and this remains to be determined.

The Roundup®-induced Ca^{2+} overload and cell death observed in rat testis were mediated by L-VDCC, IP_3- and ryanodine-mediated Ca^{2+} release, clearly indicating that disruption in Ca^{2+} homeostasis plays a critical role in the toxic effects of this herbicide. More than two decades ago, Olorunsogo [57] have demonstrated that glyphosate increased mitochondrial membrane permeability to protons and Ca^{2+}, early suggesting a mechanism of the toxic effect of this herbicide. Calcium may enter the cell through plasma membrane channels following an extracellular signal, or be released from the endoplasmic reticulum into the cytosol in response to intracellular messengers. Imbalance of these events can lead to Ca^{2+} overload, one of the earlier steps for eliciting oxidative stress and cellular apoptosis. The normal function of the endoplasmic reticulum is essential for Ca^{2+} signaling, and disturbance of Ca^{2+} homeostasis may affect protein folding and induce endoplasmic reticulum stress [58,59].

The Ca^{2+}-mediated Roundup® cytotoxicity in rat testis also involves the activation of kinase cascades including PLC/PKC, PI3K, ERK1/2 and p38MAPK. These kinases might be associated with the adaptative response to endoplasmic reticulum stress and/or ROS generation within the testis. Eukaryotic cells respond to extracellular stimuli by recruiting signal transduction pathways, including those involved in Ca^{2+} homeostasis. Signaling pathways orchestrated by MAPK family members (ERK, p38MAPK and SAPK/JNK) have been associated with hypo and hyperthyroidism in rat testis [37,38]. However, their physiological roles and regulation are not completely understood. Although architecturally homologous to the Ras/MAPK pathway, the SAPK/JNK and p38MAPK pathways are not primarily activated by mitogens, but by cellular stress (such as oxidative stress) and inflammatory cytokines, resulting in growth arrest, apoptosis, or activation of immune cells. Moreover, it has been suggested that p38MAPK pathway function primarily inhibiting cell growth and promoting either necrotic or apoptotic cell death [60]. Interestingly, under our experimental conditions, apoptotic cell death was not associated with acute Roundup® toxicity, taking into account that the caspase 3 activity was reduced after Roundup® exposure (results not shown), suggesting that apoptosis could not be a response to an acute insult at low doses of the pesticide. Also, hyperphosphorylation of p38MAPK may activate the nuclear factor erytroid 2-related factor 2 (NRF2), a ROS-activated signal transduction molecule, which can modulate genes encoding the enzymes involved in the antioxidant defense system. Increased p38MAPK phosphorylation associated with shift of NRF2 into the nuclear fraction as well as modulation of antioxidant and proinflammatory signaling pathways were recently demonstrated in Sertoli cells [61].

The pro-oxidant potential of long term exposure to Roundup® has been largely demonstrated in different non-target organisms, such as fish [62,63] and mammals, leading to hepatotoxicity, nephrotoxicity, lipid peroxidation, and genotoxicity [64]. Also, exposure of a human keratinocyte cell line to glyphosate for 30 min to 24 h evidenced cytotoxic effects, concomitant with oxidative disorders [65]. Accordingly, supplementation with vitamin C or E decreased lipid peroxidation in Roundup®-treated keratinocytes [14,19], as well as after exposure to other organophosphate pesticides [66]. In line with this, we found that ascorbic acid (vitamin C) and Trolox® (an analogue of vitamin E) abolished the Roundup®-induced Ca^{2+} influx and necrosis in the testis and in Sertoli cells in culture corroborating a role for depleted antioxidant defenses in the Roundup® cytotoxicity.

The Sertoli cell antioxidant system is characterized by relatively high activity of SOD, CAT, GPx, GST, GR, γGT, catalase and intracellular GSH levels [37,38,67,68]. The induction of oxidative stress in testicular cells by Roundup® was confirmed by decreased GSH levels accompanied by increased TBARS and protein carbonyl contents, indicating a potential risk for oxidative damage in Roundup® treated testis, linking our findings with ROS overgeneration and oxidative damage. Enhancement of TBARS levels by Roundup® in rat testis, suggests lipid peroxidation, a process of oxidative degradation of polyunsaturated fatty acids resulting in impaired membrane structure and function. Levels of lipid peroxidation and protein carbonyls, indicating possible damage to lipids and proteins, respectively, may be used as biomarkers of herbicide exposure [35,69,70]. Therefore, TBARS and protein carbonyls could be due to the concomitant increase in ROS generation and depleted antioxidant defense systems in Roundup® treated testicular cells.

In line with this, pesticide exposure induced the enzymatic activities of GPx, GR, GST, GGT, CAT, SOD and G6PD that could support GSH depletion. GSH plays an essential role in the testis, providing a defense system against oxidant agents. The complex redox system of GSH consists of GPx, GR, GST and γGT. The redox cycling of oxidized glutathione is catalyzed by GR, whereas the supply of NADPH, a major reducing agent for this redox cycling, is provided by G6PD activity [31]. Moreover, GSH conjugates xenobiotics by generating a biotransformation product through a reaction catalyzed by GST [31,71-73]. Considering that this GSH conjugation is one of the first steps in xenobiotic detoxification, the GSH depletion observed in our experimental conditions may be a consequence of its consumption by GST activation. Glutathione metabolism can also be modulated by the ecto-γGT, which breaks it down, generating a γ-glutamyl amino acid and cysteinyl-glycine to the GSH synthesis. Thus, γGT causes the degradation of extracellular GSH to provide cells with substrates for *de novo* GSH synthesis [31]. Our results are in agreement with previous studies reporting a GSH depletion in response to other pesticides and herbicides in different cellular populations, *in vitro* [14,74,75], and *in vivo* [76].

Moreover, the membrane-bound enzyme γGT may be used as a biomarker of Sertoli cell toxicity. Also, testicular cell exposure to the pesticide Roundup® stimulated the activity of other antioxidant enzymes such as CAT and SOD. CAT and GPx are both involved in removing H_2O_2; however, they must co-operate with other defense systems such as SOD, to render the cells more resistant to oxidative damage [31,77,78]. Taking into account these findings, alterations in the activity of the enzymes involved in GSH metabolism induced by Roundup® may reflect a declined cellular defense against oxidative damage within the testis. Alterations in such antioxidants have been used as an index of unbalanced ROS generation and oxidative stress in physiological systems [79].

The enzyme γGT is primarily involved in metabolizing extracellular GSH, providing precursor amino acids to be assimilated and reutilized for intracellular GSH synthesis [80]. However, although the γGT activity was increased in Roundup®-treated testis, results showed low cytoplasmic GSH levels, which were probably due to downregulation of the Na^+-coupled ^{14}C-MeAIB accumulation in testicular cells. It is feasible that the decreased ^{14}C-MeAIB uptake could diminish the amino acid availability for GSH *de novo* synthesis. Therefore, the decreased GSH levels after exposure to Roundup® might be due either to its consumption by conjugation *via* GST activity and/or to its decreased synthesis/turnover.

Conclusion:

Recent reports demonstrate that many currently used pesticides have the capacity to disrupt reproductive function in animals. Although this reproductive dysfunction is typically characterized by alterations in serum steroid hormone levels, disruptions in spermatogenesis, and loss of fertility, the mechanisms involved in pesticide-induced infertility remain unclear. Particularly, the herbicide Roundup® has been described as environmental endocrine disruptor by inhibiting the steroidogenic acute regulatory (StAR) protein expression in Leydig cells (Walsh et al 2000). Interestingly, glyphosate alone did not alter steroid production, indicating that at least another component of the formulation is required to disrupt steroidogenesis [12]. Our present findings shed light into additional mechanisms beyond the classical ones, which can contribute for understanding the possible effects of glyphosate/Roundup on the decline of male reproductive functions. We suggest that Ca^{2+}-mediated toxicity, oxidative imbalance and disrupted signaling mechanisms seem to underlie the acute exposure to low doses of glyphosate-Roundup® in prepubertal rat testis, being the Sertoli cells one of the targets for this pesticide. Considering that normal onset of spermatogenesis depends on Sertoli cell function to support and nourish germ cells, the impairment of these cells may affect male fertility. Altogether, the Ca^{2+}-mediated disturbances by glyphosate/Roundup® in rat testicular cells around 36 ppm, at levels below those to which people working with this herbicide are typically exposed, could contribute to the reproductive outcomes induced by this formulation observed in male agricultural workers exposed to this pesticide in prepubertal age.

Declaration of interest

Authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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References

[1] FIOCRUZ (Fundação Oswaldo Cruz). Agência Fiocruz de notícias: Saúde e ciência para todos. Filme sobre uso de agrotóxico será exibido nos 57 anos da Ensp. Publicada em 09-09-2011. Disponível em: http://www.fiocruz.br/ccs/cgi/cgilua.exe/sys/start.htm?infoid=4211&sid=3

[2] Cox, C. Glyphosate (Roundup). J. Pest. Reform. 18:3–17; 1998.

[3] Williams, G. M.; Kroes, R.; Munro, I. C. Safety evaluation and risk assessment of the herbicide Roundup and its active ingredient, glyphosate, for human. Regul. Toxicol. Pharmacol. 31:117–65; 2000.

[4] Marc, J.; Mulner-Lorillon, O.; Boulben, S.; Hureau, D.; Durand, G.; Bellé, R. Pesticide Roundup provokes cell division dysfunction at the level of CDK1/cyclin B activation. Chem. Res. Toxicol. 15;326–331; 2002.

[5] Benachour, N.; Sipahutar, H.; Moslemi, S.; Gasnier, C.; Travert, C.; Seralini, G. E. Time and dose-dependent effects of Roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53:126-133; 2007.

[6] El-Shenawy, N. S. Oxidative stress responses of rats exposed to Roundup and its active ingredient glyphosate. Environ. Toxicol. Pharmacol. 28:379–385; 2009.

[7] Tsui, M. T.; Chu, L. M. Aquatic toxicity of glyphosatebased formulations: comparison between different organisms and the effects of environmental factors. Chemosphere 52:1189–1197; 2003.

[8] Richard, S.; Moslemi, S.; Sipahutar, H.; Benachour, N.; Séralini, G. E. Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environ. Health Perspect. 113:716–720; 2005.

[9] Benachour, N.; Séralini, G. E. Glyphosate Formulations Induce Apoptosis and Necrosis in Human Umbilical, Embryonic, and Placental Cells. Chem. Res. Toxicol. 22:97–105; 2009.

[10] Romano, R. M.; Romano, M. A.; Bernardi, M. M.; Oliveira, C. A.; Furtado, P. V. Prepubertal exposure to commercial formulation of the herbicide glyphosate alters testosterone levels and testicular morphology. Arch Toxicol. 84:309-317; 2010.

[11] Mesnage, R.; Bernay, B.; Séralini, G. E. Ethoxylated adjuvants of glyphosate-based herbicides are active principles of human cell toxicity. Toxicology. 2012 doi:pii: S0300-483X(12)00345-9. 10.1016/j.tox.2012.09.006

[12] Walsh, L. P.; McCormick, C.; Martin, C.; Stocco, D. M. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environ. Health Perspect. 108:769–776; 2000.

[13] Peixoto, F. Comparative effects of the Roundup and glyphosate on mitochondrial oxidative phosphorylation. Chemosphere. 61:1115-1122; 2005.

[14] Gehin, A.; Guyon, C.; Nicod, L. Glyphosate-induced antioxidant imbalance in HaCaT: The protective effect of Vitamins C and E. Environ Toxicol Pharmacol. 22:27-34; 2006.

[15] Gasnier, C.; Dumont, C.; Benachour, N.; Clair, E.; Chagnon, M. C.; Seralini, G. E. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology. 262:184-191; 2009.

[16] Clair, E.; Mesnage, R.; Travert, C.; Séralini, G. E. A glyphosate-based herbicide induces necrosis and apoptosis in mature rat testicular cells in vitro, and testosterone decrease at lower levels. Toxicol. in Vitro. 26:269-279; 2012.

[17] Kim, Y. H.; Hong, J. R.; Gil, H. W.; Song, H. Y.; Hong, S. Y. Mixtures of glyphosate and surfactant TN20 accelerate cell death via mitochondrial damage-induced apoptosis and necrosis. Toxicol In Vitro. 27:191-197; 2013.

[18] Gong, Y.; Wu, J.; Huang, Y.; Shen, S.; Han, X. Nonylphenol induces apoptosis in rat testicular Sertoli cells via endoplasmic reticulum stress. Toxicol Lett. 186:84-95; 2009.

[19] Gehin, A.; Guillaume, Y. C.; Millet, J.; Guyon, C.; Nicod, L. Vitamins C and E reverse effect of herbicide-induced toxicity on human epidermal cells HaCaT: a biochemometric approach. Int. J. Pharm. 288:219–226; 2005.

[20] Nazıroğlu, M.; Dikici, D. M.; Dursun, S. Role of oxidative stress and Ca^{2+} signaling on molecular pathways of neuropathic pain in diabetes: focus on TRP channels. Neurochem. Res. 37:2065-2075; 2012.

[21] Calì, T.; Ottolini, D.; Brini, M. Mitochondrial Ca(2+) and neurodegeneration. Cell Calcium. 52:73-85; 2012.

[22] Wiegert, J. S.; Bading, H. Activity-dependent calcium signaling and ERK-MAP kinases in neurons: a link to structural plasticity of the nucleus and gene transcription regulation. Cell Calcium. 49:296-305; 2011.

[23] Whorton, D.; Krauss, R. M.; Marshall, S.; Milby, T. H. Infertility in male pesticide workers. Lancet 805:1259–1261; 1977.

[24] Yousef, M. I.; Salem, M. H.; Ibrahim, H. Z.; Helmi, S.; Seehy, M. A.; Bertheussen, K. Toxic effects of carbofuran and glyphosate on semen characteristics in rabbits. J. Environ. Sci. Health B. 30:513–34; 1995.

[25] Schettler, T.; Solomon, G.; Kaplan, J.; Valenti, M. Generations at risk: how environmental toxicants may affect reproductive health in California. Cambridge: MIT Press Ltd; 2003.

[26] Bretveld, R.; Brouwers, M.; Ebisch, I.; Roeleveld, N. Influence of pesticides on male fertility. Scand. J. Work Environ. Health 33:13-28; 2007.

[27] Hokanson, R.; Fudge, R.; Chowdhary, R.; Busbee, D. Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate. Human. Experim. Toxicol. 26:747–752; 2007.

[28] Dallegrave, E.; Mantese, F. D.; Oliveira, R. T.; Andrade, A. J.; Dalsenter, P. R.; Langeloh, A. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch. Toxicol. 81:665-673; 2007.

[29] Séralini, G. E.; Clair, E.; Mesnage, R.; Gress, S.; Defarge, N.; Malatesta, M.; Hennequin, D.; de Vendômois, J. S. Long term toxicity of a Roundup herbicide and a Roundup-tolerant genetically modified maize. Food Chem. Toxicol. 50:4221-31; 2012.

[30] Shiogiri, N. S.; Paulino, M. G.; Carraschi, S. P.; Baraldi, F. G.; da Cruz, C.; Fernandes, M. N. Acute exposure of a glyphosate-based herbicide affects the gills and liver of the Neotropical fish, Piaractus mesopotamicus. Environ. Toxicol. Pharmacol. 34:388-396; 2012.

[31] Halliwell, B.; Gutteridge, J. M. C., 4 ed. Free Radicals in Biology and Medicine. New York: Oxford Biosciences; 2007.

[32] Padron, O. F.; Brackett, N. L.; Sharma, R. K.; Kohn, S.; Lynne, C. M.; Thomas, A. J. Jr.; Agarwal, A. Seminal reactive oxygen species, sperm motility and morphology in men with spinal cord injury. Fertil. Steril. 67:1115–1120; 1997.

[33] Sikka, S. C. Relative impact of oxidative stress on male reproductive function. Curr. Med. Chem. 8:851–862; 2001.

[34] Agarwal, A.; Saleh, R.A.; Bedaiwy, M. A. Role of reactive oxygen species in the pathophysiology of human reproduction. Fertil. Steril. 79:829-843; 2003.

[35] Menezes, C. C.; Fonseca, M. B.; Loro, V. L. Santi, A.; Cattaneo, R.; Clasen, B.; Pretto, A.; Morsch, V. M. Roundup effects on oxidative stress parameters and recovery pattern of Rhamdia quelen. Arch. Environ. Contam. Toxicol. 60:665-671; 2011.

[36] Romero, D. M.; Molina, M. C. R, Juárez, A. B. Oxidative stress induced by commercial glyphosate formulation in a tolerant strain of Chlorella kessleri. Ecotoxicol. Environ. Saf. 74:741-747; 2011.

[37] Zamoner, A.; Barreto, K. P.; Filho, D. W.; Sell, F.; Woehl, V. M.; Guma, F. C.; Silva, F. R. M. B.; Pessoa-Pureur, R. Hyperthyroidism in the developing rat testis is associated with oxidative stress and hyperphosphorylated vimentin accumulation. Mol. Cell. Endocrinol. 267:116-126; 2007.

[38] Zamoner, A.; Barreto, K. P.; Filho, D. W.; Sell, F.; Woehl, V. M.; Guma, F. C.; Silva, F. R. M. B.; Pessoa-Pureur, R. Propylthiouracil-induced congenital hypothyroidism upregulates vimentin phosphorylation and depletes antioxidant defenses in immature rat testis. J. Mol. Endocrinol. 40:125-135; 2008.

[39] Dorrington, J. H.; Roller, N. F.; Fritz, I. B. Effects of follicle-stimulating hormone on cultures of Sertoli cell preparations. Mol. Cell. Endocrinol. **3:**57-70; 1975.

[40] Galdieri, M.; Ziparo, E.; Palombi, F.; Russo, M.; Stefanini, M.; Pure Sertoli cell cultures: a new model for the study of somatic-germ cell interactions. J. Androl. 5:249-254; 1981.

[41] Bouraïma-Lelong, H.; Vanneste, M.; Delalande, C.; **Zanatta, L.;** Wolczynski, S.; Carreau, S. Aromatase gene expression in immature rat Sertoli cells: age-related changes in the FSH signaling pathway. Reprod. Fertil. Dev. 22:508-515; 2010.

[42] Zamoner, A.; Royer, C.; Barreto, K. P.; Pessoa-Pureur, R.; Silva, F. R. M. B. Ionic involvement and kinase activity on the mechanism of nongenomic action of thyroid hormones on $^{45}Ca^{2+}$ uptake in cerebral cortex from young rats. Neurosci. Res. 57:98–103; 2007.

[43] Lowry, O. H.; Rosenbrough, N. J.; Farr, A. L.; Randall, R. J. Protein measurement with folin phenol reagent. J. Biol. Chem. 193:265-267; 1951.

[44] Silva, F.R.; Leite, L.D.; Barreto, K.P.; D'Agostini, C.; Zamoner, A. Effect of 3,5,3'-triiodo-L-thyronine on amino acid accumulation and membrane potential in Sertoli cells of the rat testis. Life Sci. 69:977-986; 2001.

[45] Orlowsky, M.; Meister, A. Gamma-glutamyl-p-nitroanilide: a new convenient substrate for determination and study of l- and D-gamma-glutamyltranspeptidase activities. Biochim. Biophys. Acta, 73:679–68; 1963.

[46] Aebi, H. Catalase in vitro. Methods Enzymol. 204:234–54; 1984.

[47] Misra, H. P.; Fridovich, I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J. Biol. Chem. 247:188–192; 1972.

[48] Boveris, A.; Fraga, C. G.; Varsavsky, A. I.; Koch, O. R. Increased chemiluminescence and superoxide production in the liver of chronically ethanol-treated rats. Arch. Biochem. Biophys. 227:534–541; 1983.

[49] Flohé, L.; Günzler, W. A. Assays of glutathione peroxidase. Methods Enzymol. 105:114–121; 1984.

[50] Calberg, I.; Mannervik, B. Glutathione reductase from rat liver. Methods Enzymol. 113:484–490; 1985.

[51] Habig, W. H.; Pabst, M. J.; Jacoby, W. B. Glutathione S-transferases: the first enzymatic step in mercapturic acid formation. J. Biol. Chem. 249:7130–7139; 1976.

[52] Beutler, E.; Duron, O.; Kelly, B. M. Improved method for the determination of blood glutathione. J. Lab. Clin. Med. 61:882–890; 1963.

[53] Bird, R. P.; Draper, A. H. Comparative studies on different methods of malondyhaldehydedetermination. Methods Enzymol. 90:105–110; 1984.

[54] Levine, R. L.; Garland, D.; Oliver, C. N.; Amici, A.; Climent, I.; Lenz A. G.; Ahn, B. W.; Shaltiel, S.; Stadtman, E. R. Determination of carbonyl content in oxidatively modified proteins. Methods Enzymol. 186:464–478; 1990.

[55] Oliveira, A. G; Telles, L. F; Hess, R. A; Mahecha, G. A.; Oliveira, C. A. Effects of the herbicide Roundup on the epididymal region of drakes Anas platyrhynchos. Reprod. Toxicol. 23:182-191; 2007.

[56] Romano, M. A.; Romano, R. M.; Santos, L. D.; Wisniewski, P.; Campos, D. A.; Souza, P. B.; Viau, P.; Bernardi, M. M.; Nunes. M. T.; Oliveira, C. A. Glyphosate

impairs male offspring reproductive development by disrupting gonadotropin expression. Arch. Toxicol. 86:663–673; 2012.

[57] Olorunsogo, O. O. Modification of the transport of protons and Ca^{2+} ions across mitochondrial coupling membrane by N-(phosphonomethyl)glycine. Toxicology. 61:205-209; 1990.

[58] High, S.; Lecomte, F. J. L.; Russell, S. J.; Abell, B. M.; Oliver, J. D. Glycoprotein folding in the endoplasmic reticulum: a tale of three chaperones? FEBS Letters, 476:38–41; 2000.

[59] Bánhegyi, G.; Baumeister, P.; Benedetti, A.; Dong, D.; Fu, Y.; Lee, A. S.; Li, J.; Mao, C.; Margittai, E.; Ni, M.; Paschen, W.; Piccirella, S.; Senesi, S.; Sitia, R.; Wang, M.; Yang, W. Endoplasmic reticulum stress. Ann. N. Y. Acad. Sci. 1113:58-71; 2007.

[60] Kyriakis, J. M, Avruch, J. Sounding the alarm: protein kinase cascades activated by stress and inflammation. J. Biol. Chem. 271:24313-24316; 1996.

[61] Xiao, Y.; Karnati, S.; Qian, G.; Nenicu, A.; Fan, W.; Tchatalbachev, S. Höland, A.; Hossain, H.; Guillou, F.; Lüers, G. H.; Baumgart-Vogt, E. Cre-mediated stress affects sirtuin expression levels, peroxisome biogenesis and metabolism, antioxidant and proinflammatory signaling pathways. PLoS One. 7:e41097; 2012.

[62] Cattaneo, R.; Clasen, B.; Loro, V.L,; de Menezes, C. C.; Pretto, A.; Baldisserotto, B.; Santi, A.; de Avila, L. A. Toxicological responses of Cyprinus carpio exposed to a commercial formulation containing glyphosate. Bull Environ. Contam. Toxicol. 87:597-602; 2011.

[63] Guilherme, S.; Gaivão, I.; Santos, M. A.; Pacheco, M. DNA damage in fish (Anguilla anguilla) exposed to a glyphosate-based herbicide -- elucidation of organ-specificity and the role of oxidative stress. Mutat. Res. 743:1-9; 2012.

[64] Cavuşoğlu, K.; Yapar, K.; Oruç, E.; Yalçın, E. Protective effect of Ginkgo biloba L. leaf extract against glyphosate toxicity in Swiss albino mice. J Med. Food. 14:1263-1272; 2011.

[65] Elie-Caille, C.; Heu, C.; Guyon, C.; Nicod, L. Morphological damages of a glyphosate-treated human keratinocyte cell line revealed by a micro- to nanoscale microscopic investigation. Cel.l Biol. Toxicol. 26:331-339; 2010.

[66] Ahmed, R. S.; Seth, V.; Pasha, S. T.; Banerjee, B. D. Influence of dietary ginger (Zingiber officinales Rosc) on oxidative stress induced by malathion in rats. Food Chem. Toxicol. 38:443-450; 2000.

[67] Bauché, F.; Fouchard, M. H.; Jégou B. Antioxidant system in rat testicular cells. FEBS Lett., 349:392–396; 1994.

[68] Aly, H. A.; Khafagy, R. M. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-induced cytotoxicity accompanied by oxidative stress in rat Sertoli cells: Possible role of mitochondrial fractions of Sertoli cells. Toxicol. Appl. Pharmacol. 252:273-280; 2011.

[69] Sayeed, I.; Parvez, S.; Pandey, S.; Bin-Hafeez, B.; Rizwanul, H.; Raisuddin, S. Oxidative stress biomarkers of exposure to deltamethrin in freshwater fish, Channa punctatus Bloch. Ecotoxicol. Environ .Saf. 56:295–301; 2003.

[70] Almroth, B. C.; Sturve, J.; Berglund, A.; Forlin, L. Oxidative damage in eelpout (Zoarces viviparous), measured as protein carbonyl and TBARS, as biomarkers. Aquat. Toxicol. 73:171–180; 2005.

[71] Bray, T. M.; Taylor C. G. Tissue glutathione, nutrition, and oxidative stress. Can. J. Physiol. Pharmacol. 71:746–751; 1993.

[72] Dringen, R. Metabolism and functions of glutathione in brain. Prog. Neurobiol. 62:649-71; 2000.

[73] Hellou, J.; Ross, N. W.; Moon, T. W. Glutathione, glutathione S-transferase, and glutathione conjugates, complementary markers of oxidative stress in aquatic biota. Environ. Sci. Pollut. Res. Int.19:2007-2023; 2012.

[74] Cereser, C.; Boget, S.; Parvaz, P.; Revol A. Thiram-induced cytotoxicity is accompanied by a rapid and drastic oxidation of reduced glutathione with consecutive lipid peroxidation and cell death. Toxicology. 163:153-162; 2001.

[75] Bukowska, B. Effects of 2,4-d and its metabolite 2,4-dichlorophenol on antioxidant enzymes and level of glutathione in human erythrocytes. Comp. Biochem. Phys. C, 135:435-41; 2003.

[76] Banerjee, B. D.; Seth, V.; Bhattacharya, A.; Pasha, S. T.; Chakraborty A. K. Biochemical effects of some pesticides on lipid peroxidation and free-radical scavengers. Toxicol. Lett. 107:33-47; 1999.

[77] Yusa, T.; Crapo, J. D.; Freeman, B. A. Liposome-mediated augmentation of brain SOD and catalase inhibits CNS O_2 toxicity. J. Appl. Physiol. 57:1674-1681; 1984.

[78] de Haan, J. B.; Cristiano, F.; Iannello, R.; Bladier, C.; Kelner, M. J.; Kola, I. Elevation in the ratio of Cu/Zn-superoxide dismutase to glutathione peroxidase activity induces features of cellular senescence and this effect is mediated by hydrogen peroxide. Hum. Mol. Genet. 5:283-292; 1996.

[79] Matés, J. M.; Sánchez-Jiménez, F. M. Role of reactive oxygen species in apoptosis: implications for cancer therapy. Int. J. Biochem. Cell. Biol. 32:157-170; 2000.

[80] Lee, D. H.; Blomhoff, R.; Jacobs, D. R. Jr. Is serum gamma glutamyltransferase a marker of oxidative stress? Free Radic. Res. 38:535-539; 2004.

Legends

Figure 1. Dose-response curve of Roundup® on $^{45}Ca^{2+}$ uptake (A) and on LDH release (B) on immature rat testis. Rat testis were pre-incubated for 15 min and then incubated in the presence of 0.1 mCi/mL of $^{45}Ca^{2}$ with or without Roundup® at different concentrations: 0.72 to 360 ppm, corresponding to 0.00072 to 0.36 g/L . Values are means ± S.E.M. of 8 animals. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with control group.

Figure 2. Involvement of calcium channels (VDCCs), intracellular calcium levels and kinase pathways on $^{45}Ca^{2+}$ uptake in immature rat testis. Testes were pre-incubated for 15 min with or without 10 μM nifedipine (L-VDCC blocker), or 50 μM Bapta-AM (intracellular calcium chelator), or 50 μM dantrolene (ryanodine calcium channel blocker) (**A**), or 10 μM H89 (PKA inhibitor), or 10 μM U73122 (PLC inhibitor), or 20 μM RO 31-8220 (PKC inhibitor) (**B**), or 10 μM SB 239063 (p38 MAPK inhibitor), or 10 μM LY 294002 (PI3K inhibitor) or 10 μM PD 98059 (MAPK inhibitor) (**C**). After that, the testes were incubated with or without 36 ppm (0.036 g/L) Roundup® (A, B and C) for 30 min (incubation) in the presence of 0.1 mCi/mL of $^{45}Ca^{2+}$. Instead of Roundup®, testes were incubated with glyphosate (0.036 g/L) after pre-incubation with 10 μM nifedipine (**D**). Values are means ± S.E.M. of 8 animals expressed as percentage of control. $*p < 0.01$ compared with control group. $^{\#}p < 0.01$ compared with Roundup® or glyphosate group.

Figure 3. Effect of Roundup® on ERK1/2 **(A)** and p38^{MAPK} **(B)** activation in rat testis. After 15 min pre-incubation, rat testes were incubated with Roundup® (36 ppm or 0.036 g/L during 30 min). Tissue was lysed and the total and phosphorylated levels of ERK1/2 and p38^{MAPK} were determined by Western blot. Data are reported as means SEM of 6 animals in each group and expressed as % of control. Statistically significant differences as determined by one-way ANOVA followed by Tukey-Kramer multiple comparison test are indicated: ***P<0.0001 compared with control group. Representative immunoblots were shown in the insets. P-ERK = phospho-ERK; Pp38 = phospho-p38.

Figure 4. Involvement of oxidative stress on Roundup®-induced $^{45}Ca^{2+}$ uptake and cell death in rat testis. Testes were pre-incubated for 20 min in the presence or absence of the antioxidants 100 μM Trolox® or 100 μM ascorbic acid. After that, the tissue was incubated with or without 36 ppm or 0.036 g/L Roundup® plus the antioxidants for 30 min. Data are reported as means ± SEM of 8 animals in each group and expressed as % of control. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test are indicated: *P< 0.05 and **P< 0.01 compared with control group. $^{\#}$P< 0.05 compared with Roundup® group.

Figure 5. Effect of Roundup® on lipid peroxidation and protein carbonyl levels in immature rat testis. Testis were incubated in the presence or absence of 36 ppm or 0.036 g/L Roundup® for 30 min. Data from thiobarbituric acid-reactive substances (TBARS) measurement of lipid peroxidation and protein carbonyl levels are reported as means ±

S.E.M. of 6 animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. *P<0.01, ***P<0.0001.

Figure 6. Effect of Roundup on glutathione levels (GSH) and on the activities of glucose-6-phosphate dehydrogenase (G6PD), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione S-transferase (GST), gamma-glutamyl transferase (γGT), catalase (CAT) and superoxide dismutase (SOD) in immature rat testis. Rat testes were incubated for 30 min in the presence or absence of 36 ppm or 0.036 g/L Roundup®. Data are reported as means ± S.E.M. of 8 animals from each group. Statistically significant differences from controls, as determined by Student's *t* test, are indicated. *P<0.01, ***P<0.0001.

Figure 7. Effect of Roundup® on ^{14}C-MeAIB accumulation in rat testes. Rat testes were incubated for 30 min in the presence of 3.7 kBq/mL ^{14}C-MeAIB 3.7 kBq/mL with or without 36 ppm or 0.036 g/L Roundup®. Data are reported as means ± S.E.M. of 8 animals from each group. Statistically significant difference from control, as determined by Student's t test, is indicated. *P<0.001.

Figure 8. Dose-response curve of Roundup® on $^{45}Ca^{2+}$ uptake (A) and on LDH release (B) on Sertoli cell culture from immature rat testis. Sertoli cells were pre-incubated for 15 min and then incubated for 15 or 30 min in the presence of 0.1 mCi/mL of $^{45}Ca^{2}$ with or without 36 ppm or 0.036 g/L Roundup® at different concentrations (0.72 to 360

ppm). Values are means ± S.E.M. of four independent experiments. *p<0.01, ***p<0.0001 compared with control group.

Figure 9. Prevention of antioxidants on Roundup®-induced $^{45}Ca^{2+}$ uptake and cell death in Sertoli cells from immature rat testis. Sertoli cells were pre-incubated for 20 min in the presence or absence of 100 µM Trolox®, or vitamin C (100 µM or 200 µM), or vitamin C plus Trolox® (75 µM vit C + 50 µM Trolox or 150 µM vit C + 75 µM Trolox). After that, the tissue was incubated with or without 36 ppm or 0.036 g/L Roundup® in the presence or not of the antioxidants for 30 min. Values are means ± S.E.M. of four independent experiments. Statistically significant differences from controls, as determined by one-way ANOVA followed by Bonferroni multiple comparison test are indicated: *P< 0.01 and ***P< 0.0001 compared with control group. #P< 0.05 compared with Roundup® group.

Figure 10. Proposed mechanism of reproductive toxicity of Roundup® to Sertoli cells. Roundup® increased intracellular Ca^{2+} concentration by opening voltage-dependent calcium channels (VDCC) and endoplasmic reticulum receptors (such as IP_3 and ryanodine) leading to Ca^{2+}overload within the cells, which set off oxidative stress and cell death. Activation of Ca^{2+} influx and necrotic cell death are dependent on PLC/PKC, PI3K and MAPK signaling pathways. PLC/IP_3 together with ryanodine Ca^{2+} channels promote Ca^{2+} release from the endoplasmic reticulum, contributing to Ca^{2+} overloading. Roundup® exposure induced the activity of the following antioxidant enzymes: glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GST), gamma-glutamyl transferase (γGT), catalase (CAT), superoxide dismutase

(SOD), glucose-6-phosphate dehydrogenase (G6PD). Considering that conjugation with GSH is one of the first steps in detoxifying a xenobiotic, the GSH depletion may occur as a consequence of its consumption by the activation of GST. The GSH metabolism can also be modulated by the γGT, which breaks it down generating a γ-glutamyl amino acid and cysteinyl-glycine to the GSH synthesis. Altogether, these events suggest that Roundup® compromises the Sertoli cell antioxidant defense system, probably leading to endoplasmic reticulum stress and cell death which may affect male reproduction. (R= Roundup®)

Figure 1

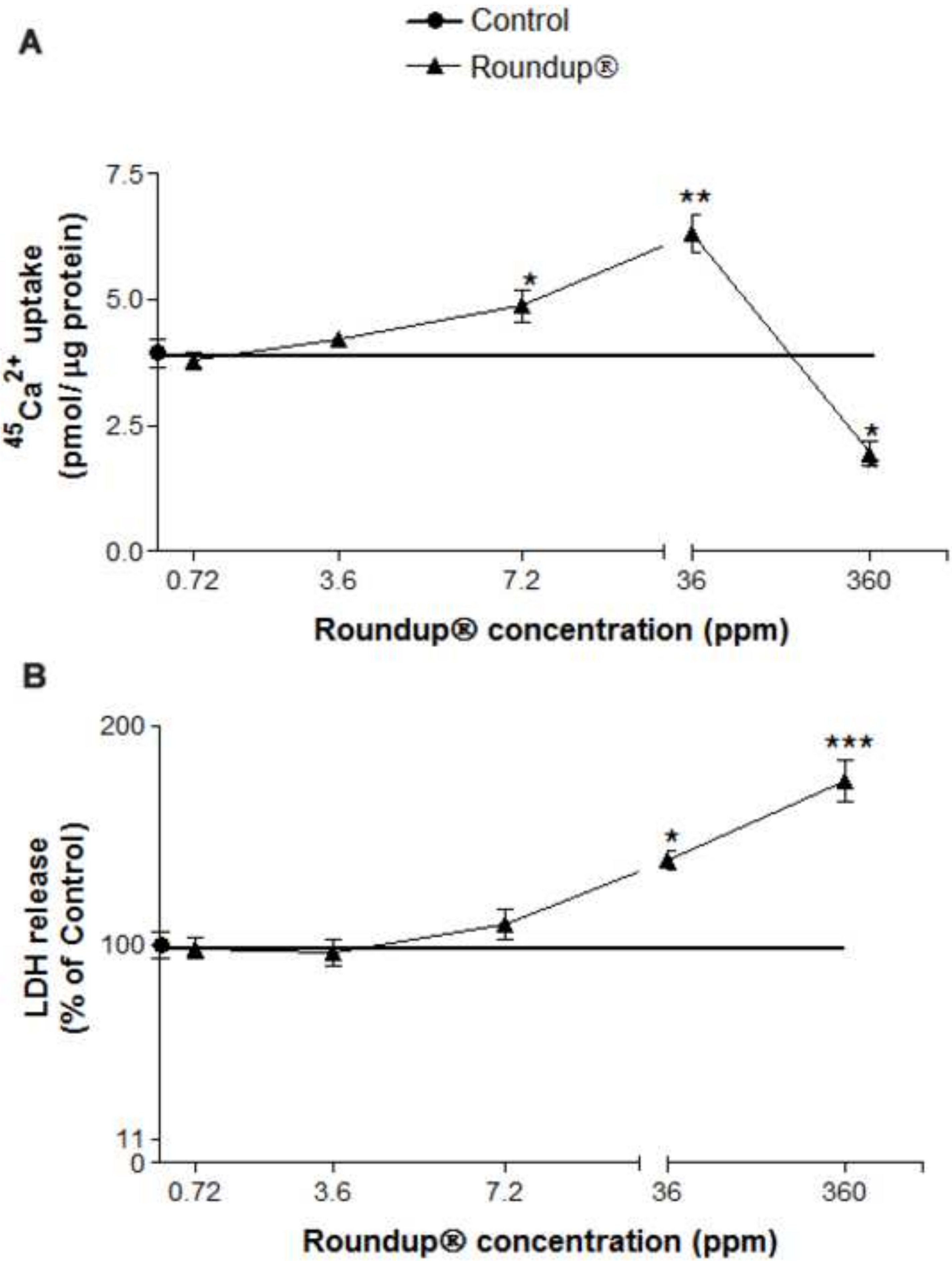

A
Control
Roundup®
7.5
5.0
2.5
0.0
$^{45}Ca^{2+}$ uptake (pmol/µg protein)
**
*
*
0.72
3.6
7.2
36
360
Roundup® concentration (ppm)
B
200
100
11
0
LDH release (% of Control)
***
*
0.72
3.6
7.2
36
360
Roundup® concentration (ppm)

Figure 2

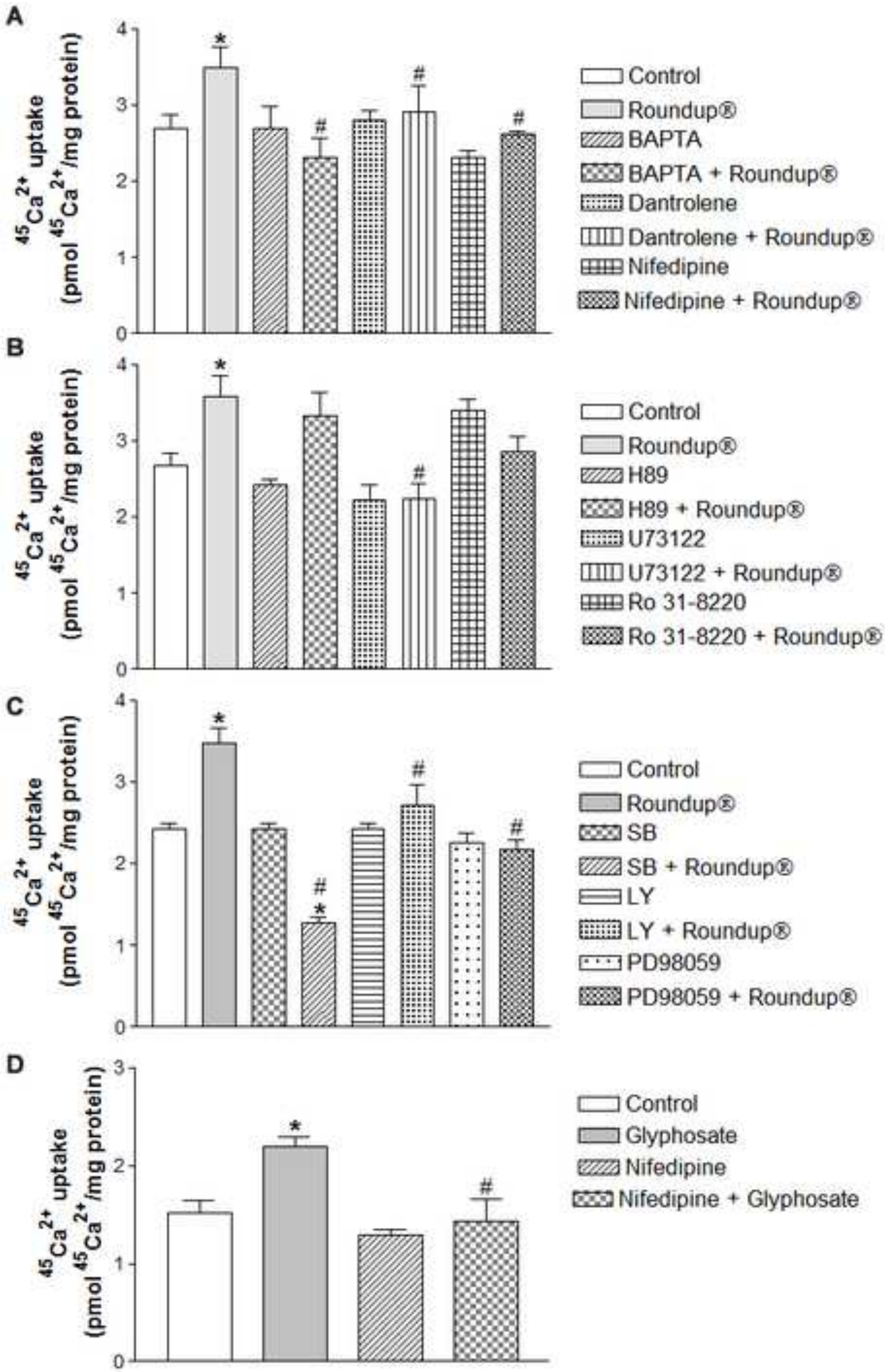

A
B
C
D
$^{45}Ca^{2+}$ uptake (pmol $^{45}Ca^{2+}$/mg protein)
Control
Roundup®
BAPTA
BAPTA + Roundup®
Dantrolene
Dantrolene + Roundup®
Nifedipine
Nifedipine + Roundup®
H89
H89 + Roundup®
U73122
U73122 + Roundup®
Ro 31-8220
Ro 31-8220 + Roundup®
SB
SB + Roundup®
LY
LY + Roundup®
PD98059
PD98059 + Roundup®
Glyphosate
Nifedipine + Glyphosate

Figure 3

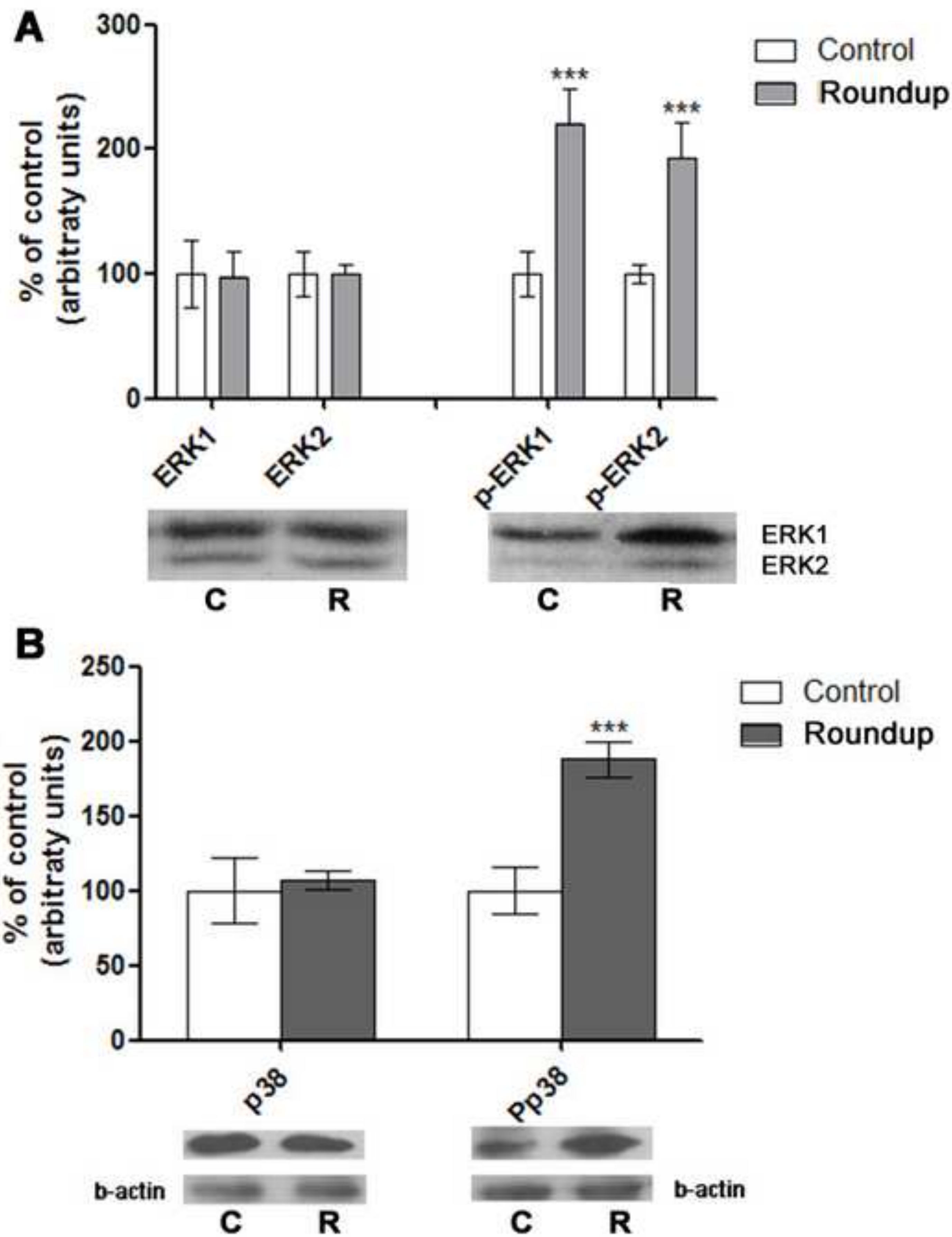
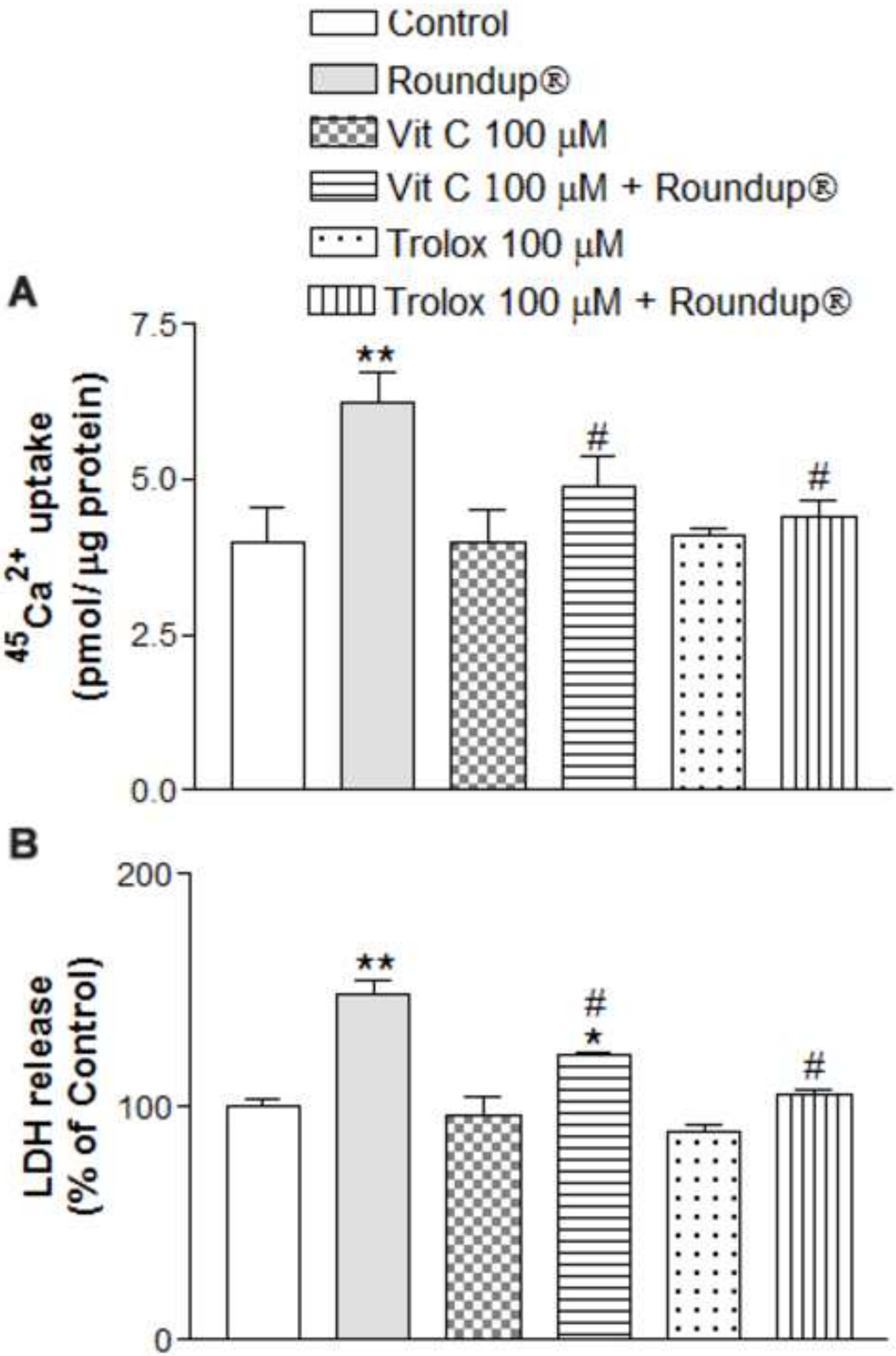

A
300
200
100
0
% of control
(arbitraty units)
Control
Roundup
***
***
ERK1
ERK2
p-ERK1
p-ERK2
ERK1
ERK2
C
R
C
R
B
250
200
150
100
50
0
% of control
(arbitraty units)
Control
Roundup
***
p38
Pp38
b-actin
b-actin
C
R
C
R

Figure 4


Control
Roundup®
Vit C 100 µM
Vit C 100 µM + Roundup®
Trolox 100 µM
Trolox 100 µM + Roundup®
A
45Ca2+ uptake (pmol/µg protein)
7.5
5.0
2.5
0.0
**
#
#
B
LDH release (% of Control)
200
100
0
**
#
*
#

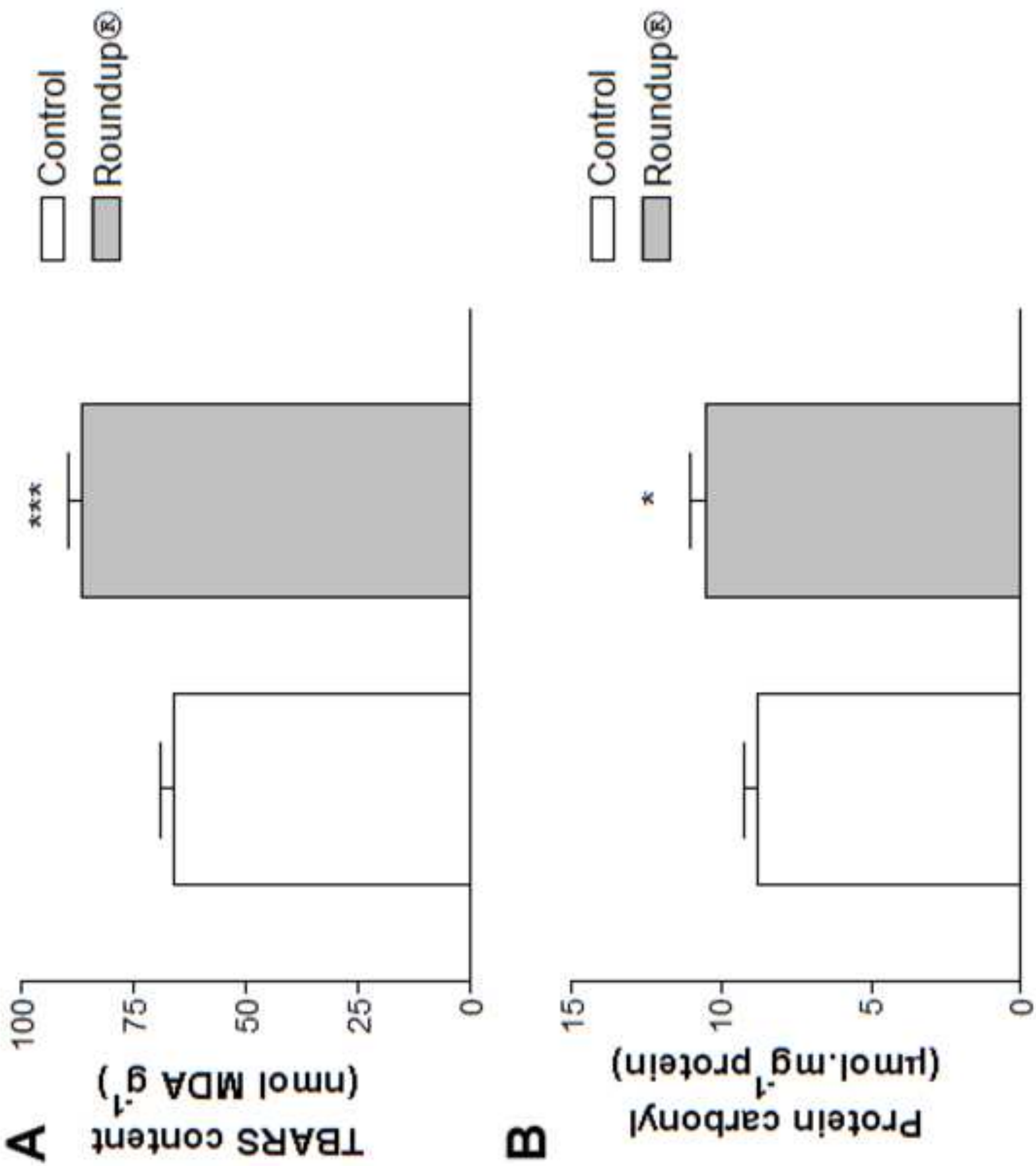

A
TBARS content
(nmol MDA g^{-1})
100
75
50
25
0
***
Control
Roundup®
B
Protein carbonyl
(µmol.mg^{-1} protein)
15
10
5
0
*
Control
Roundup®

Figure 5

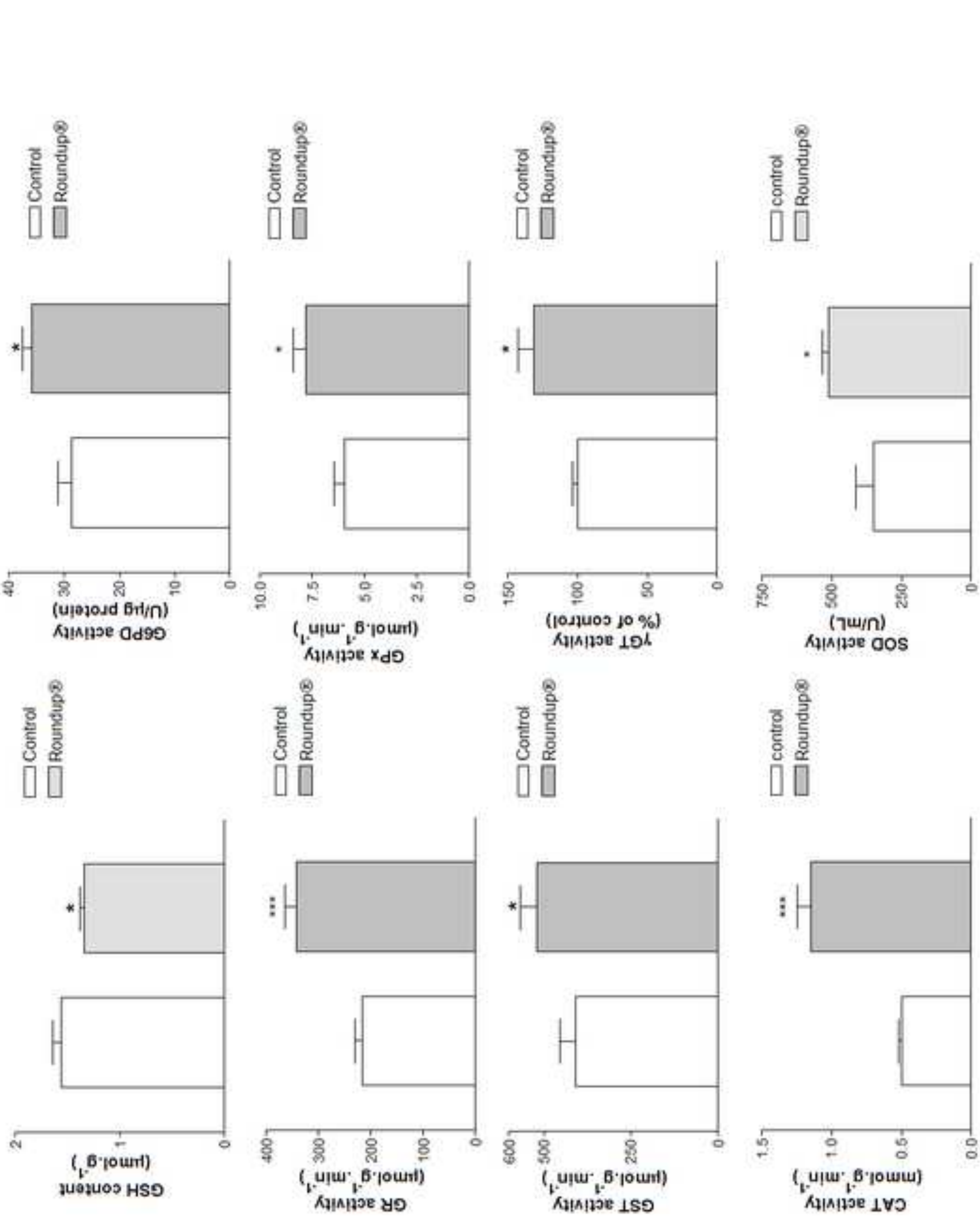

GSH content (µmol.g-1)
GR activity (µmol.g-1.min-1)
GST activity (µmol.g-1.min-1)
CAT activity (mmol.g-1.min-1)
G6PD activity (U/µg protein)
GPx activity (µmol.g-1.min-1)
γGT activity (% of control)
SOD activity (U/mL)
Control
Roundup®
control

Figure 6

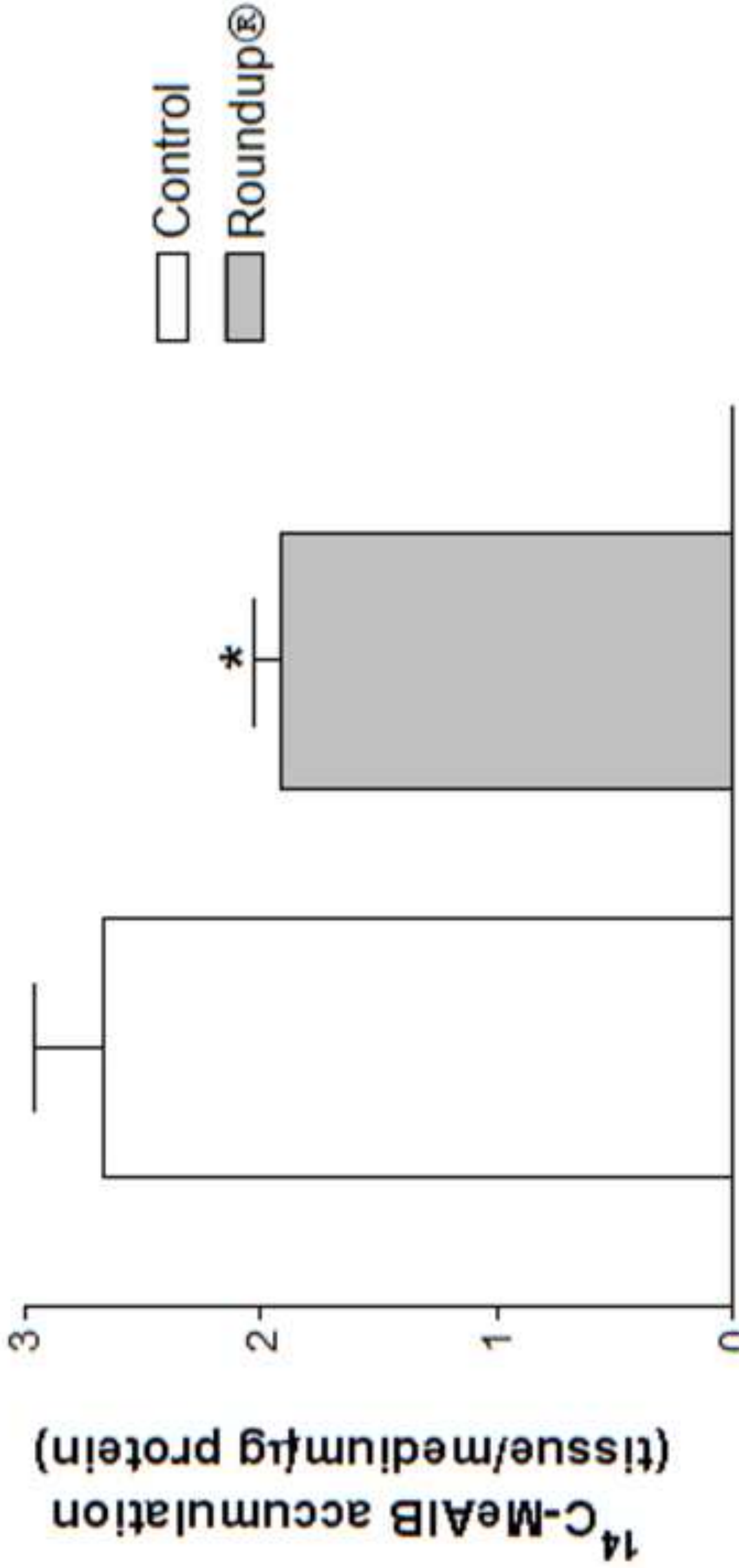
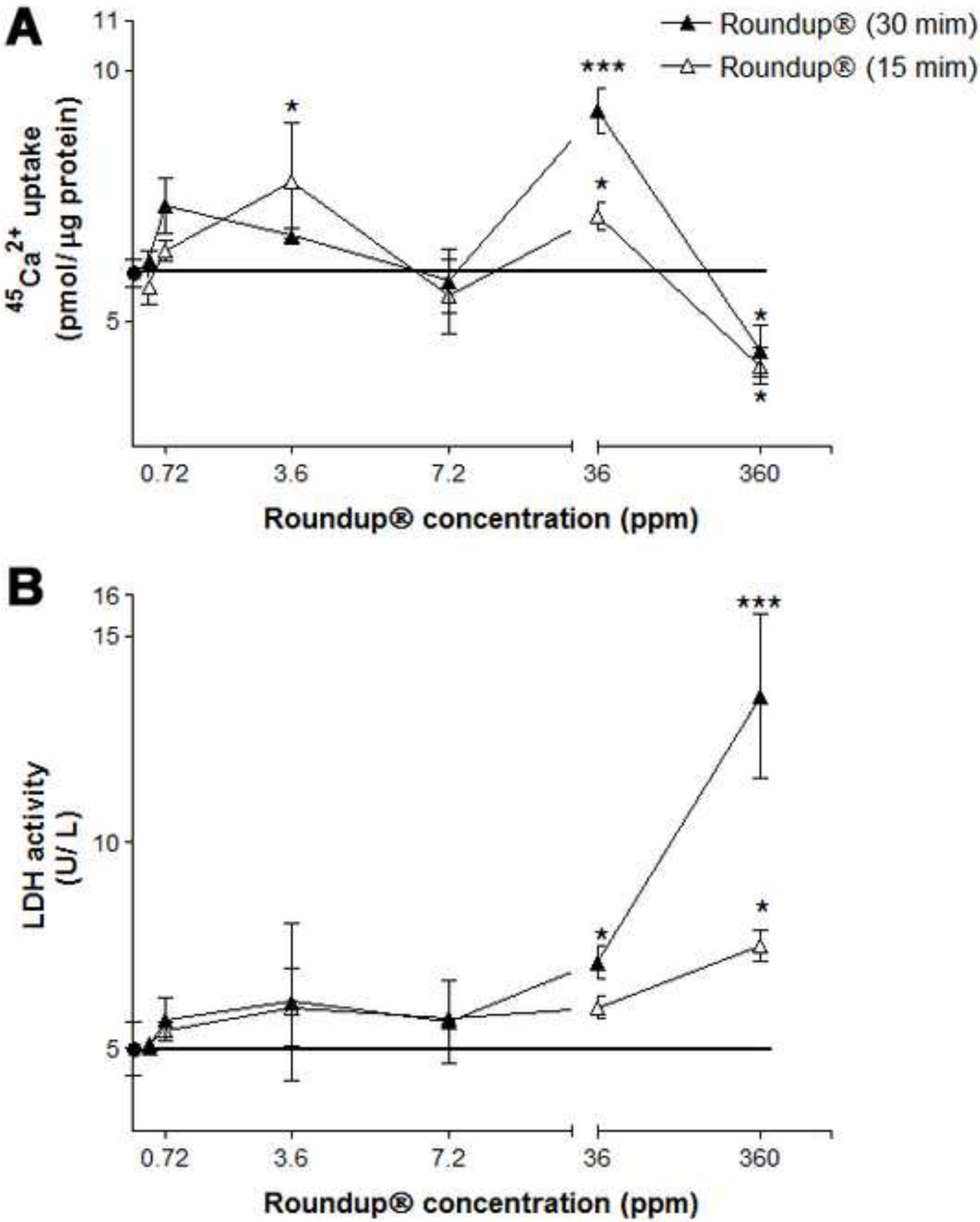

Control
Roundup®
*
0
1
2
3
^{14}C-MeAIB accumulation
(tissue/medium/µg protein)

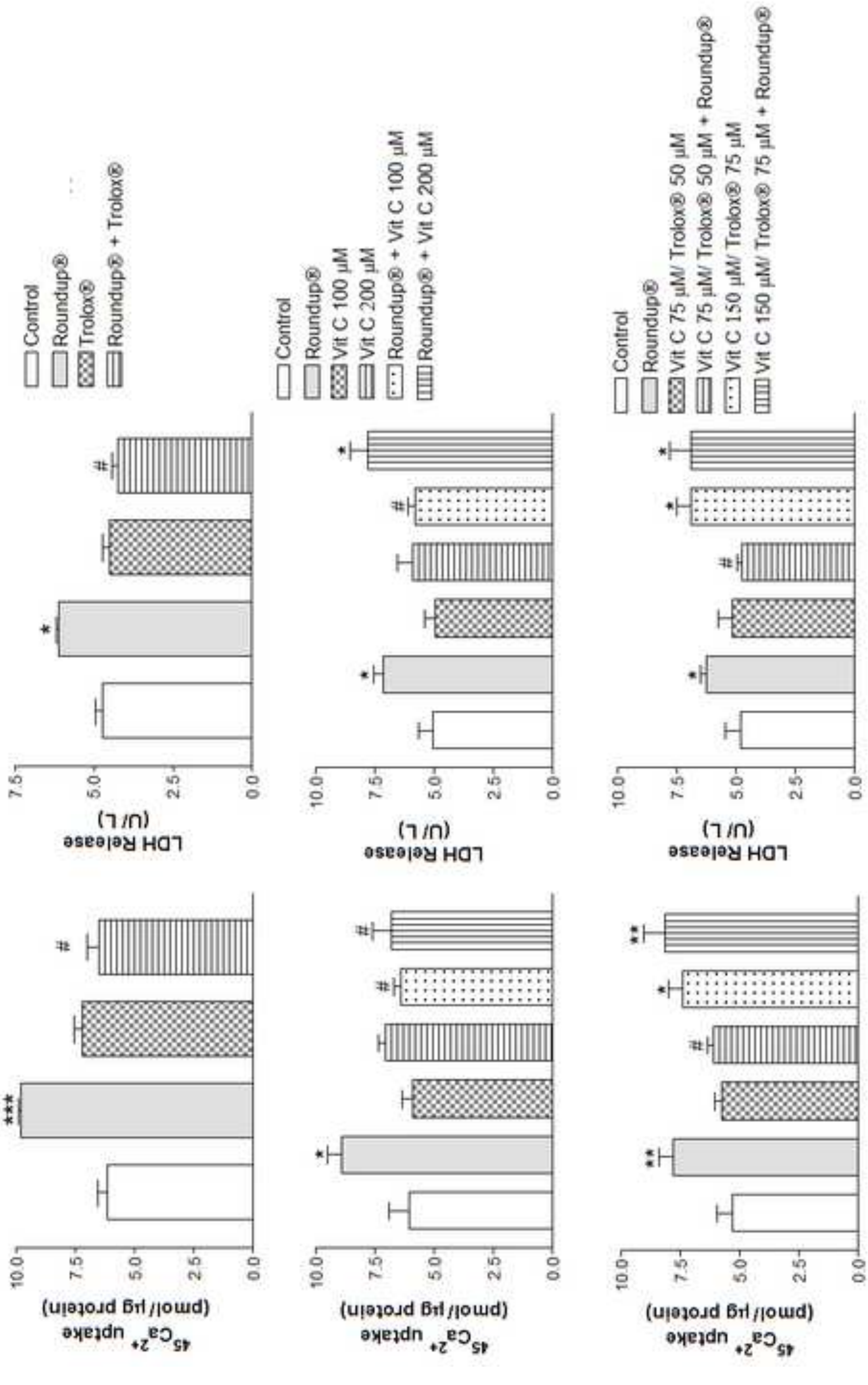
Figure 7

Figure 8

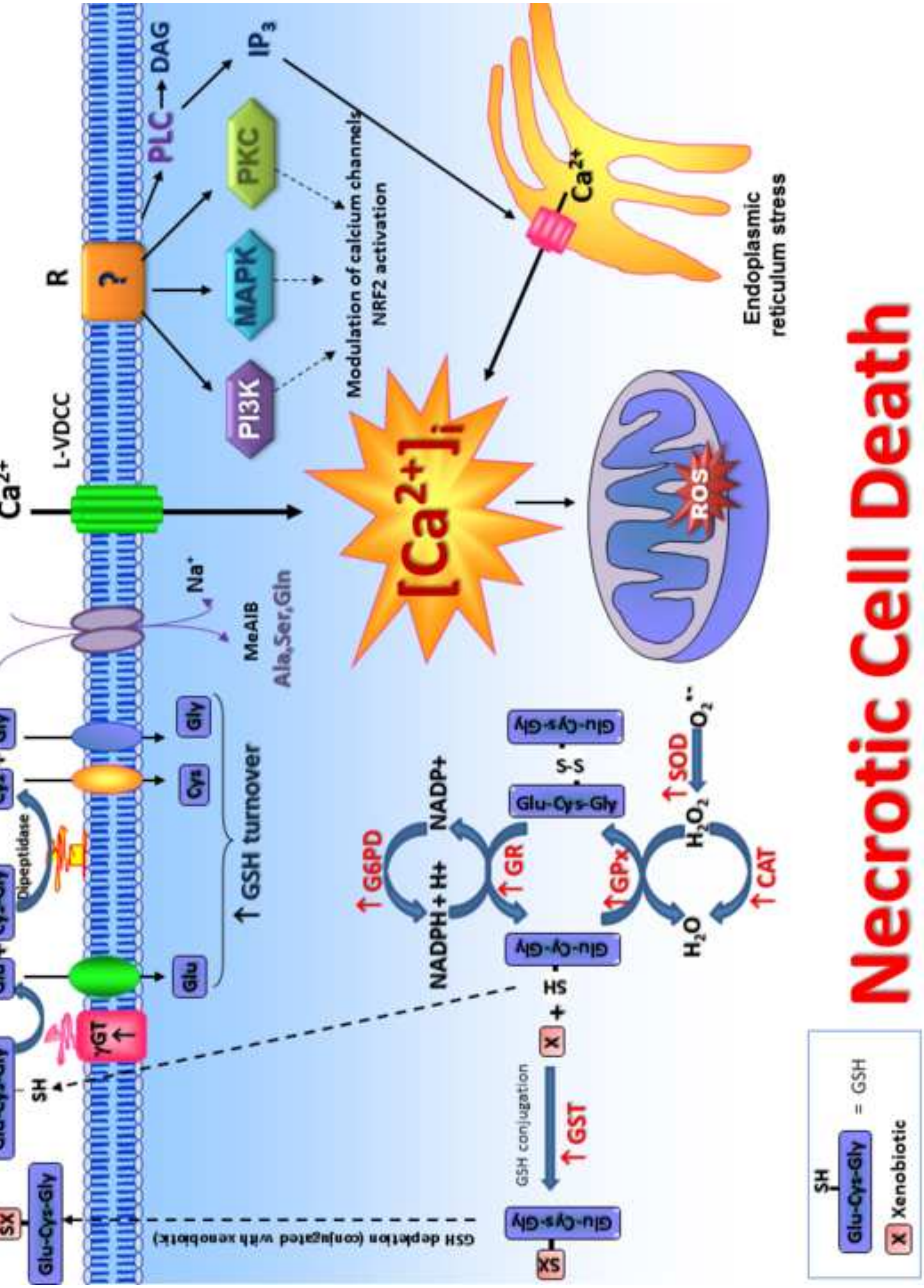


A
B
Roundup® (30 mim)
Roundup® (15 mim)
$^{45}Ca^{2+}$ uptake (pmol/ µg protein)
11
10
5
***
*
0.72
3.6
7.2
36
360
Roundup® concentration (ppm)
LDH activity (U/ L)
16
15
10
5

Figure 9

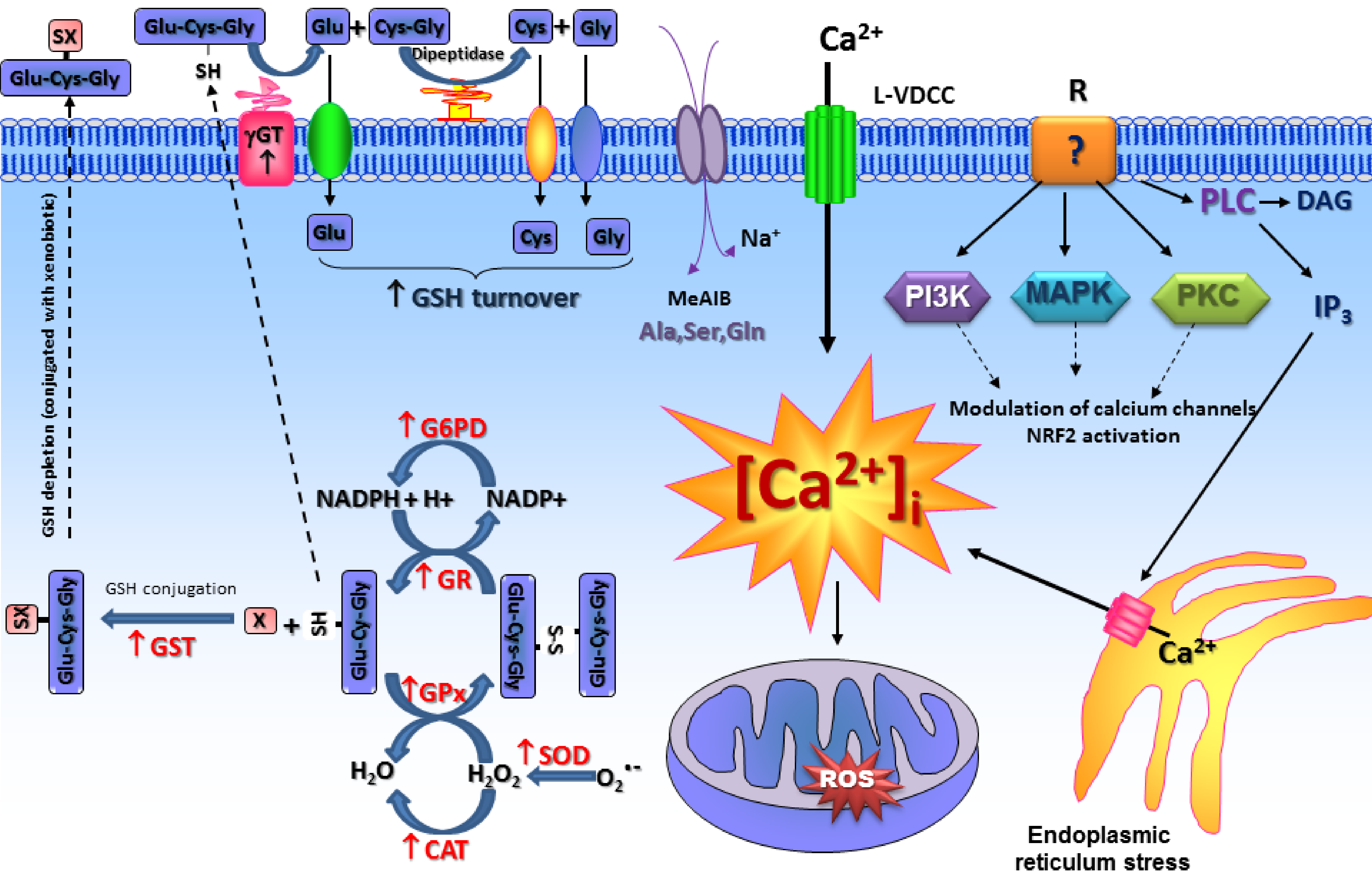


45Ca2+ uptake (pmol/ µg protein)
LDH Release (U/ L)
Control
Roundup®
Trolox®
Roundup® + Trolox®
Vit C 100 µM
Vit C 200 µM
Roundup® + Vit C 100 µM
Roundup® + Vit C 200 µM
Vit C 75 µM/ Trolox® 50 µM
Vit C 75 µM/ Trolox® 50 µM + Roundup®
Vit C 150 µM/ Trolox® 75 µM
Vit C 150 µM/ Trolox® 75 µM + Roundup®



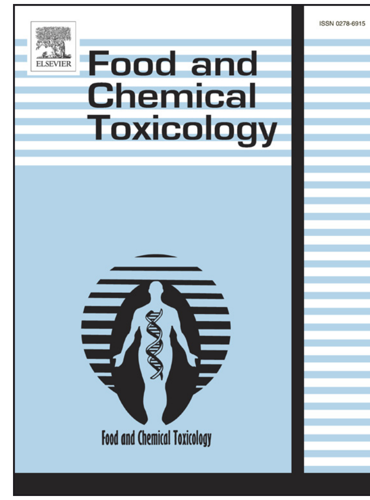
Ca2+
L-VDCC
R
?
PLC → DAG
IP3
PI3K
MAPK
PKC
Modulation of calcium channels
NRF2 activation
[Ca2+]i
Ca2+
Endoplasmic reticulum stress
ROS
Necrotic Cell Death
Na+
MeAIB
Ala,Ser,Gln
Glu-Cys-Gly
SH
γGT
Glu
Cys-Gly
Dipeptidase
Cys
Gly
↑ GSH turnover
SX
GSH depletion (conjugated with xenobiotic)
GSH conjugation
↑ GST
X
HS
Glu-Cy-Gly
NADPH + H+
NADP+
↑ G6PD
↑ GR
↑ GPx
S-S
H2O
H2O2
O2•-
↑ SOD
↑ CAT
= GSH
Xenobiotic

Figure 10



Necrotic Cell Death

Accepted Manuscript



Glyphosate induces human breast cancer cells growth via estrogen receptors

Siriporn Thongprakaisang, Apinya Thiantanawat, Nuchanart Rangkadilok, Tawit Suriyo, Jutamaad Satayavivad

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Glyphosate induces human breast cancer cells growth via estrogen receptors

Siriporn Thongprakaisang[1], Apinya Thiantanawat [2,3], Nuchanart Rangkadilok[1,3], Tawit Suriyo[3], Jutamaad Satayavivad[1,3,4 *]

[1] Environmental Toxicology Program and [2]Applied Biological Sciences Program, Chulabhorn Graduate Institute, Kamphaengphet 6 Road, Laksi, Bangkok 10210, Thailand.

[3] Laboratory of Pharmacology, Chulabhorn Research Institute, Kamphaengphet 6 Road, Laksi, Bangkok 10210, Thailand.

[4] Center of Excellence on Environmental Health and Toxicology, Office of the Higher Education Commission, Ministry of Education, Bangkok, 10400, Thailand.

* Corresponding author: Assoc. Prof. Jutamaad Satayavivad

Laboratory of Pharmacology

Chulabhorn Research Institute

Kamphaengphet 6 Road, Laksi

Bangkok 10210, Thailand.

Telephone: 66-2-5740622 ext. 3917

Fax: 66-2-5742027

Email: jutamaad@cri.or.th

Glyphosate induces human breast cancer cells growth via estrogen receptors

1. Introduction

Glyphosate, *N*-(phosphonomethyl) glycine, is widely used as an active ingredient of herbicide products to control weeds in cropped and non-cropped fields around the world. In addition, glyphosate formulations have been used extensively in genetically modified glyphosate-resistant plants (Acquavella et al., 2004). The herbicidal activity of glyphosate is rather specific on the targets with the inhibition of the shikimate pathway which only presents in plants and micro-organisms (Solomon et al., 2007). Glyphosate is considered as a non toxic herbicide because of its low LD_{50} (the concentration that caused 50% deaths); > 4 g/kg (WHO, 1994). However, the reproductive toxicities of glyphosate have been extensively studied in both animals and human. Up to now, the endocrine disrupting effects of glyphosate were not observed in the *in vivo* but the *in vitro* studies and the epidemiological studies have still conflicted in those findings due to their differences in the experimental designs, methodology and confounding factors (Brake and Evenson, 2004; Dallegrave et al., 2007; Daruich et al., 2001; Mandel et al., 2005; Marc et al., 2004; McDuffie et al., 2001). The synergistic effects of glyphosate and surfactants in its herbicide formulations have been concerned especially the endocrine disrupting activity (Richard et al., 2005). Most studies found that the adjuvants or surfactants in most formulations were more toxic and could enhance the toxic effects of glyphosate (Gasnier et al., 2009; Marc et al., 2004; Walsh et al., 2000). Glyphosate at concentrations used in agriculture (21-42 mM) was found to be toxic to human embryonic and placental cells (Benachour et al., 2007; Richard et al., 2005). Roundup®, a popular formulation could disrupted the synthesis of hormones in the mouse MA-10 Leydig tumor cell line (Benachour et al., 2007; Walsh et al., 2000). Glyphosate has been shown to disrupt the animal cell cycle in urchin eggs based on its surfactant carrying in commercial formulation (Marc et al., 2004). Recently, it was reported that at lower non toxic concentrations of Roundup and glyphosate (< 1 ug/L), the main endocrine disruption is a testosterone decrease by 35%. Most potential adverse health effects were reported on the commercial glyphosate formulations. The expression of estrogen-regulated genes relating to tumor formation and tumor growth in hormone dependent human breast cancer MCF-7 cells were reported to be disrupted (Hokanson et al., 2007). Furthermore, synergistic effects between glyphosate and estrogen (17β-estradiol or E2) have been demonstrated. Glyphosate was reported to have a disrupting effect on estrogen receptor alpha (ERα) and beta (ERβ) transcriptional activities in HepG2 cells transiently transfected with ERE-TK-Luciferase and androgen receptor (AR) in MDA-MB453-kb2 cells (Gasnier et al., 2009). These toxic effects were reported to be more frequent with glyphosate-based herbicides than that with glyphosate alone.

This present study aims to evaluate the estrogenic effects of glyphosate alone at the range of concentrations that has been reported in environmental conditions and exposed human. Estrogenic and/or antiestrogenic effects of glyphosate were investigated and compared with endogeneous estrogen in the estrogen dependent human breast cancer cells T47D. Since glyphosate-based herbicides have been used intensively in soybean cultivation and soybean also contains the phytoestrogen, genistein, the interactive effects of these two compounds were also studied.

2. Materials and Methods

2.1 Chemicals and reagents

Glyphosate (>98%) was purchased from AccuStandard (New Haven, CT, USA). 17β-estradiol (E2) was obtained from Sigma-Aldrich (St. Louis, MO, USA). ICI 182780 and genistein was purchased from Tocris Bioscience (Ellisville, MS, USA). All the other reagents and chemicals were of analytical grade and obtained from commercial sources.

2.2 Cell lines and culture conditions

A hormone-dependent human breast cancer, T47D, a stably ERE-luc construct transfected hormone-dependent breast cancer, T47D-KBluc, and a hormone-independent human breast cancer, MDA-MB231, were purchased from the American Type Culture Collection (ATCC) (Rockville, MD, USA). T47D and T47D-KBluc cells were maintained in recommended standard medium of RPMI 1640 supplemented with 10% fetal bovine serum (FBS) (JR Scientific, Woodland, CA, USA), 4.5 g/L D-glucose, 1 mM sodium pyruvate, 2 mM glutamine, 100 U/mL penicillin/ streptomycin (P/S) and 8 mg/L insulin. MDA-MB-231 cells were cultured in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/mL penicillin/ streptomycin (P/S) and 1% non-essential amino acid. All cells were cultured in a humidified incubator with 5%CO_2 and 95% air at 37 °C. Culture medium and supplements were purchased from Gibco-Invitrogen Life Technology (Carlbad, CA, USA).

2.3 In vitro estrogen receptor activation-reporter assay

In order to study the estrogenicity and/or antiestrogenicity effect of glyphosate, the T47D-KBluc cell; stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct, was used in this study (Wilson et al., 2004). To minimize the effect of estrogen in the medium, five days prior to the assay, cells were switched to grow in a non-phenol red RPMI modified medium with a replacement of 10% FBS to 10% dextran-charcoal treated FBS (CSS) (HyClone, South Logan, UT, USA), together with all other supplements except penicillin/ streptomycin. One day prior to the assay, cells were seeded at

10^4 cells/100µL/well in 96-well luminometer plates (Corning Incorporated, Corning, NY, USA) and were allowed to attach overnight. Dosing media was further modified by reduction to 5% CSS. Media was then replaced with 100 µL/well of dosing media in which the final concentration of glyphosate ranged from 10^{-12} to 10^{-6} M. The same range of estradiol (E2) concentrations was used as the positive control agonist for estrogen receptor activation. The dosing media was used as the negative control and wells without cells were used as blank. After 24 h incubation, cells were washed with 100 µL phosphate buffered saline (Sigma-Aldrich, St Louis, MO, USA) at room temperature, then harvested in 25 µL lysis buffer (Promega, Madison, WI, USA). The luciferase assay was performed by injecting 50 µL of reaction buffer (25 mM glycylglycine, 15 mM $MgCl_2$, 5 mM ATP, 0.1 mg/mL BSA, pH 7.8) and 50 µL of 1 mM D-luciferin (Promega, Madison, WI, USA) by using microplate luminometer (SpectraMax L, Molecular Devices, Sunnyvale, CA, USA) and fluorescent intensity was measured. The luciferase activity was quantified as relative light units (RLU).

2.4 Cell viability MTT assay

Cell growth and viability were tested using the 3-(4,5-dimetylthiazol,2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma-Aldrich, St. Louis, MO, USA) reagent assay. Cells were seeded at 10^4 cells/100µL/well in 96-well microtiter plates. For the E2 withdrawal condition, cells were cultured in 10% CSS and a non phenol red RPMI medium containing all supplemented reagents for 4-days before seeding. After 24 h incubation for the attachment, the cells were treated with varying concentrations of E2 or glyphostae ranging from 10^{-12} to 10^{-6} M. In the present of E2 receptor antagonist condition, E2- or glyphosate-treated cells were co-incubation with ICI 182780 (1 and 10 nM). After 24 hr incubation period, the medium was removed and 10 µL of MTT [5mg/mL in phosphate-buffered saline (PBS)] in 90 µL medium was added into each well. Cells were further incubated for 4h, then the medium was removed and 100 µL dimetyl sulfoxide (Merck, Whitehouse Station, NJ, USA) was added to each well to dissolve precipitated dye. The optical density was read at 570 nm / 650 nm using microplate readers (SpectraMax Plus 384, Molecular Devices, Sunnyvale, CA, USA). Cell sensitivity to a chemical was expressed as the % growth compared to the control (vehicle treated) cells.

2.5 Western blot analysis

Whole-cell extracts were prepared from cells treated for 6 and 24 h with 10^{-12}, 10^{-9}, and 10^{-7} M glyphosate and non-treated control in two medium conditions, completed medium, and hormone-withdrawal medium by lysis of cold PBS-washed cells in lysis buffer [50 mM Tris-HCl (Sigma, USA), 150 mM NaCl (Sigma, USA), 1% Triton-X (Merck, USA), 1 mM EDTA (Merck, USA), 1mM sodium orthovanadate (Na_3VO_4, Sigma, USA), 100 mM sodium fluoride (NaF, Sigma, USA), protease cocktail inhibitor (Calbiochem, Germany) and 0.1 mM phenylmethylsulfonyl fluoride (PMSF, Calbiochem, Germany). The lysates were then sonicated, incubated on ice for 30 min, and supernatants were collected from centrifugation at 14,000 rpm for 30 min at 4°C. The lysates were either processed or stored at -80 °C until use. The protein concentration was measured using Bradford reagent (Bio-Rad Laboratories, Hercules, CA, USA). Each lysate was aliquot for an equal amount of protein, 30 μg, before mixing with Laemmli loading buffer (62.5mM Tris-HCl, 25% glycerol, 2% SDS, 0.01% bromophenol red, pH 6.8, containing 5% 2-mercaptoethanol), and then boiled at 95°C for 5 min. These samples were resolved over 7.5% polyacrylamide-SDS gels using a Mini-PROTEIN II system (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to a nitrocellulose membrane (Amersham, Arlington Heights, IL, USA) using a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad Laboratories Hercules, CA). The membrane was blocked with blocking solution (5% non-fat dry milk in 10mM Tris-HCl, pH8.0, 160mM NaCl, and 0.05% Tween-20 (USB Corporation, Cleveland, OH, USA) for 1 h at room temperature. The membrane was probed overnight with primary antibody (ERα 1:1000, ERβ 1:1000 or Beta actin 1:10,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were then washed three times, each for 10 min with Tris-Buffered Saline with 0.05% Tween-20 (TBS-T). HRP-conjugated secondary antibodies (1:3,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added to the membrane for 2 h at room temperature. The membranes were washed three times, each for 10 min with TBS-T. Protein visualization was achieved by using an enhanced chemiluminescence (ECL) (Amersham, Arlington Heights, IL, USA) and the emitted light was captured on film (Kodak Co., Rochester, NY, USA). The signals on the films were quantified using densitometer (Bio-Rad GS 710 calibrated imaging, Bio-Rad Laboratories, Hercules, CA, USA).

2.6 Cell number counting

T47D Cells were prepared in the E2 withdrawal condition 4 days before assay. Cells were placed into a 24 well culture plate (Corning Incorporated, Corning, NY, USA) with 10^4 cells/mL/well and incubated overnight in CO_2 incubator to allow cells to attach. Media was then replaced with 1 ml of treatment solution and left in CO_2 incubator for 72 h. Cells were washed with 1 ml phosphate buffered saline at room temperature and then 100 uL of a trypsin-EDTA were added to break the cells from their contacts. After trypsinization, most of the cells were removed from contact with the plate, and floated. The cell pellets were resuspended in 900 uL of basal media solution and dissociated cells by aspirating into a 5-ml syringe through a 23 ½ G needle and expelling the contents. The experiment was repeated twice and an aliquot of the cells was taken to count the number by using a counter analyzer (Z1 coulter particle counter, Beckman Coulter, Miami, FL, USA).

2.6 Statistical analysis

Data are presented as the means ± SE. Statistical significance was determined using the Student's *t*-test. A two-tailed *P* value less than 0.05 was evaluated as a statistically significant difference.

3. Results

3.1 Glyphosate induces T47D, hormone dependent breast cancer cell growth.

The hormone-dependent T47D and hormone-independent MDA-MB231 cell lines were studied both in completed medium and estrogen withdrawal medium to differentiate the effects of glyphosate from endogeneous estrogen. E2 at a concentration range from 10^{-12} – 10^{-6} M was used as a positive control. The cell viability was observed by using MTT cell viability assay. The results showed that T47D and MDA-MB231 cells exhibited different patterns of responses to glyphosate (Fig.1). Glyphosate caused the proliferative effects of T47D approximately 15 – 30 % in the absence of E2 condition (Fig.1B). This effect was about a half of E2 response which is the most potent agonist in hormone dependent ER-positive breast cancer cell. Meanwhile, glyphosate had no effect on the growth of MDA-MB231 cells both in the absence or presence of E2.

3.2 The proliferative effect of glyphosate is mediated via estrogen receptors.

Due to the fact that the proliferative effect of glyphosate occurred only in T47D cells in the absence of E2 condition, it was hypothesized that ER signaling may be involved in the glyphosate-induced proliferative effect. T47D cells were further studied using pure ER antagonist, ICI 182780, to inhibit the estrogen receptor mediated response. The effective concentration (1 nM) of ICI 182780 was added to varying concentrations of glyphosate and E2 to observe its antagonistic activity. The results showed that ICI 182780 at 1 nM mitigated the proliferative effects of both glyphosate and E2. Furthermore, higher concentration of ICI 182780 (10 nM) completely inhibited the growth promoting effects of glyphosate (Fig.2). These results suggesting that glyphosate may produce the proliferative effect via ER.

3.3. Glyphosate induces ERE-transcription activity via estrogen receptors.

We further investigated the estrogenic effect of glyphosate on ERE-transcription activity. T47D-KBluc cells, which stably transfected with a triplet ERE (estrogen response element)-promoter-luciferase reporter gene construct, were treated with the proliferative concentrations of glyphosate. The results showed that glyphosate at a concentration range from 10^{-12} to 10^{-6} M induced ERE activation 5 to 13 fold of the control and these effects were less than about a half of that induced by E2 (Fig.3A). Furthermore, glyphosate co-incubation with a pure ER antagonist, ICI 182780 exhibited the significant reduction in responses. Indeed, ICI 182780 at the concentration of 10 nM completely inhibited ERE transcriptional activity of glyphosate (Fig.3A). These results correlated with the earlier growth promotion study, confirming that glyphoste at low concentrations (10^{-12} to 10^{-6} M) produce proliferative effects in hormone dependent breast cancer cells via ER.

Since glyphosate could induce cell proliferation and ERE activation via ER, next we investigated the potential effects of glyphosate on endogenous E2 signaling. Cells were coincubated with glyphosate and E2. The results revealed that glyphosate suppressed the E2-induced ERE activation (Fig.3B). This result suggesting that in the presence of endogenous agonist (E2), glyphosate behaves as an antagonist.

3.4. Glyphosate modulates the expression of ERα and ERβ in human breast cancer cells.

We demonstrated that the induction of ERE transcription activity by glyphosate was mediated via ERs. Next, the expression of protein that involved in the classical ERs including ERα and ERβ, were studied by using western blot technique. The results demonstrated that glyphosate altered the levels of ERα and ERβ proteins (Fig. 4A-D). At 6 h of exposure, glyphosate increased the levels of both ERα and ERβ in a concentration-dependent manner while at 24 h of exposure, only ERα showed a significant induction at the highest glyphosate concentration (10^{-7} M) compared to the control group. In addition, ERβ protein levels were not changed in glyphosate-treated group when compared to the control group after 24 h of exposure. This result suggesting that glyphosate alters the expression of both ERα and ERβ in human breast cancer cells.

3.5 Interactive effects of glyphosate and phytoestrogen genistein

3.5.1 Genistein induces T47D cell proliferation and ERE activation

The phytoestrogen, genistein, is a major isoflavone found in soybeans. Genistein has a structure similar to E2 and displays estrogenic activity through ER signaling pathways (Seo et al. 2006). The results showed that genistein at a concentration range 10^{-9} - 10^{-4} M produced the concentration dependently proliferative effects (104 – 170 % of the control), with the significant effect starting from 10^{-8} M. In addition, we also found that genistein at highest tested concentration 10^{-3} M had the inhibitory effect (Fig.5A). The results were similar to previously described by Wang and his colleagues that genistein stimulated growth of MCF-7 cells at concentrations 10^{-8} – 10^{-6} M while higher concentrations (>10^{-5} M) genistein inhibited cell growth (Wang et al. 1996). Genistein also demonstrated the ability to stimulate ERE-gene transcription activity at the concentration range used in the cell viability study (Fig.5B). Genistein at the concentrations of 10^{-11} – 10^{-6} M exhibited concentration dependently ERE-activation which was aproximately 5 -25 fold of control.

3.5.2. The additive effects of genistein on glyphosate-induced ERE activation

Glyphosate is a herbicide extensively used in soybean plantations. Therefore, glyphosate has the potential to contaminate soybean products. Thus, it is interesting to evaluate whether there is an additive or synergistic effect of both compounds on the growth of cancer cells. The selection of interactive concentrations between glyphosate and genistein were based on the significant effects on the induction of ERE activity of each compound. The concentration ranges of glyphosate and genistein inducing ERE activity more than 10 fold of control included

10^{-11} to 10^{-9} M and 10^{-7} to 10^{-5} M, respectively. Actually, the concentration of glyphosate residue in soybeans should be lower than of genistein. As the information of glyphosate residues and genistein contents in soybeans were found in the range of 0.1 – 5.6 ug/g (Arregui et al., 2004; Sharma O.P., 2009) while genistein concentrations were in the range 0.01 – 1.2 mg/g (Morton et al., 1999; Murphy et al., 1999; Nakajima et al., 2005). We used this information to set the interaction model of two compounds as possible as in a real situation. The interactive levels used in this study correspond to the possible levels of glyphosate and genistein in human. Setchell and Cassidy showed that consumption of 50 mg/day of isoflavones in an adult can give rise to plasma concentrations of genistein ranging from 2.0×10^{-7} -3.2×10^{-6} M (Setchell and Cassidy, 1999), while glyphosate concentration in human body could be 1.8×10^{-8}– 1.4×10^{-6} M (Acquavella, J., 2004.) or less than 5.9×10^{-10} M (Jauhiainena, A. et al., 1991). According to these data, we had set the interaction model of these two compounds as genestine ranging from 10^{-7}-10^{-5} M and glyphosate ranging from 10^{-11}-10^{-9}. The interactive effects of glyphosate and genistein were studied by varying concentrations with fixed ratio of both as shown in Fig.6A. The results showed the significant enhancing of ERE activation in the combination of 10^{-10} M glyphosate with 10^{-6} M genistein and 10^{-9} M glyphosate with 10^{-5} M genistein.

3.5.3. The additive effects of glyphosate on genistein-induced cell proliferation

To further investigate the interactive effect on cell growth of T47D cells, glyphosate and genistein at concentrations of 10^{-9} and 10^{-7} M, respectively, were combined in E2-withdrawal condition for 72 h incubation time and cell numbers were counted as % of control (Fig.6B). This selected concentration was considered based on the equal effects of glyphosate and genistein on cell proliferation which was about 140% of the control. The results revealed that genistein at 10^{-7} M significantly enhanced the cell growth effect of 10^{-9} M of glyphosate up to 169% of control.

4. Discussion

The present study provides a better understanding of possible mechanisms underlying glyphosate toxicity in a hormone dependent human breast cancer cell. Concentrations of glyphosate tested in this study that exhibited estrogenic activity and interfered with normal estrogen signaling were relevant to the range of concentrations that has been reported in environmental conditions and exposed human. The detectable concentrations in human urines have been reported to be in the range of <0.1 - 233 ppb (<5.9 x 10^{-10} - 1.4 x 10^{-6} M) with the highest estimated systemic dose of 0.004 mg/kg (Acquavella et al., 2004 ; Jauhiainena et al., 1991).

In this study, we found that glyphosate at a log interval concentration ranging from 10^{-12} to 10^{-6} M increased the cell proliferation of a hormorne dependent breast cancer T47D cell while this effect was not observed in a hormone independent breast cancer MDA-MB231 cell. The ERE luciferase assay also supported that glyphosate behaved as a xenoestrogen to induce ERE activation because these responses can be blocked by ICI 182780, an estrogen antagonist. Although the ERs binding of glyphosate is still unknown, the ability of glyphosate to stimulate the ERE-gene transcription activity and up-regulation of ERα protein expression suggests that glyphosate may exert the stimulatory effects via the ER-dependent mechanism. As is known, ERs can bind with a wide vareity of compounds with typical structures of two hydroxyl groups separated by a rigid hydrophobic linker region and, in addition, the effective ligands possess a phenolic hydroxyl group (Ascenzi et al., 2006). Although glyphosate structure does not totally match, its responses observed in this study supported the contention that it acted like ligand binding. This unknown interaction may occur in a polar pocket at ligand binding site of ERs. Due to the hydrophilic property of glyphosate , it may access via an active phospate group. This may affect the conformation of other domains that respond to recruit other coregulators that differ from normal ER ligands. Furthermore, glyphosate also altered the levels of ER protein expression both ERα and ERβ in T47D cell at 6 h. The increased ratio of ERα / ERβ protein in the late stage, 24 h, corresponded to the observed proliferative effect of glyphosate. These results supported the finding about the regulatory role of ER β in T47D cells (Sotoca et al., 2008). They also demonstrated that the effects of estrogen like compounds on T47D cell proliferation were dependent on the actual ERα / ERβ ratio in these cells (Sotoca et al., 2008; Speirs and Walker, 2007). Glyphosate showed a different expression profile of ERα from those estrogenic stimulation induced by E2 or genistein as previously described by Seo and coworker, 2006. They showed that E2 and genistein down-regulated ERα and enhanced ERE gene expression on MCF-7 cells (Seo et al., 2006). Different cell lines have different sensitivity to estrogen, in addition, natural estrogen and estrogen-mimicking chemicals also exert a differential regulatory effect on ERα and ERβ (Cappelletti et al., 2003). This present study revealed that glyphosate treatment induced both ERs. However, patterns of ERα and ERβ induction by glyphosate were different. Glyphosate induced rapid activation of ERβ while activation of ERα was slower but prolonged. We hypothesized that glyphosate may behave like weak xenoestrogen which can activate both subtypes of ER but with a different time course.

On the other hand, our finding contradicts a recent study by Gasnier and his colleagues (Gasnier et al., 2009) who found the inhibition of the transcription activities of ERα and ERβ in HepG2 cells by Roundup® formulation, but it was not significant with pure glyphosate. This discrepancy may be due to cell types and experimental conditions. In their study, the HepG2 cells which transiently transfected with ERE-TK-Luciferase may lack some contents making it different from E2 targeted cells like breast cancer cells (Gasnier et al., 2009). Moreover, the concentrations of glyphosate in their experiments were higher than in the present study (> 10^{-5} M). Most of the studies used glyphosate-based fomulation while a few studies used pure glyphosate. Furthermore, the used concentrations were not environmentally relevant (Williams et al., 2012). Another study showed the non-estrogenic effect of glyphosate at 10^{-5} – 10^{-4} M in

MCF-7 cells (Lin and Garry, 2000), concentration ranges which cannot be compared to our study. However, the low concentration ranges should be taken into account due to many substances including pesticides and natural nutrients exerting their effects at relatively low concentrations from pico molar to micro molar (Miodini et al., 1999; Pink and Jordan, 1996; Safe and Papineni, 2006). The present study used pure glyphosate substance at log intervals from 10^{-12} to 10^{-6} M. These concentrations are in a crucial range which correlated to the potential biological levels at part per trillion (ppt) to part per billion (ppb) which have been reported in epidemiological studies (Acquavella et al., 2004; Lavy et al., 1992; Mandel et al., 2005). In this present in vitro study, we showed as estrogenicity of pure glyphosate. However, further *in vivo* study using an animal model such as a xenograft mouse model for breast cancer will confirm the present *in vitro* results and provide more physiological relevant evidence.

In addition, a single agent or chemical may exhibit a weak biological activity while mixture of compounds found environmentally could produce more noticeable effect by acting synergistically (Singleton and Khan, 2003). In fact, it has been reported that the concentrations of glyphosate in the environmental compartment and food chain are further increased due to high technology of transgenic crops and fruits demonstrating high degree of tolerance to the high levels of this compound (Solomon et al., 2007). Glyphosate-resistant soy is a popular genetically modified crop which is now becoming normal agricultural practice, thus glyphosate has higher possibility of getting into living organisms via the food chain through its application (Acquavella et al., 2004; Mandel et al., 2005). It is well known that soybean contains the phytoestrogen, genistein. Genistein acts as a weak agonist in breast tumor cells *in vitro*, it competes with E2 for binding to ERα protein, and induces activity of estrogen-responsive reporter gene constructs in the presence of ERα protein (Rajah et al., 2009). Thus, it should be of concern whether the contaminated glyphosate in soybean can interact with genistein causing alterations in their effects on the cellular system. In the present study, we showed that glyphosate had an additive effect with genistein in *in vitro* testing model. This finding should raise concern about the existence of more than one xenoestrogen such as phytoestrogen and contaminants in plant derived food which may be beneficial or harmful depending on the hormonal and pathological status of consumers. This study implied that the additive effect of glyphosate and genistein in postmenopausal woman may induce cancer cell growth. In this present *in vitro* study, we showed an estrogenicity of pure glyphosate.

In summary, we found that glyphosate exhibited a weaker estrogenic activity than estradiol. Furthermore, this study demonstrated the additive estrogenic effects of glyphosate and genistein which implied that the use of glyphosate-contaminated soybean products as dietary supplements may pose a risk of breast cancer because of their potential additive estrogenicity.

References

Acquavella, J., Alexander, B., Mandel, J., Gustin, C., Baker, B., Chapman, P., Bleeke, M., 2004. Glyphosate biomonitoring for farmers and their families: results from the farm family exposure study. Environ. Health Perspect. 112, 321-326.

Arregui, M.C., Lenardón, A., Sanchez, D., Maitre, M.I., Scotta, R., Enrique, S., 2004. Monitoring glyphosate residues in transgenic glyphosate resistant soybean. Pest Manag. Sci. 60, 163-166.

Ascenzi, P., Bocedi, A., Marino, M., 2006. Structure-function relationship of estrogen receptor alpha and beta: Impact on human health. Mol. Aspects Med. 27, 299-402.

Benachour, N., Sipahutar, H., Moslemi, S., Gasnier, C., Travert, C., Seralini, G.E., 2007. Time- and dose-dependent effects of roundup on human embryonic and placental cells. Arch. Environ. Contam. Toxicol. 53, 126-133.

Brake, D.G., Evenson, D.P., 2004. A generational study of glyphosate-tolerant soybeans on mouse fetal, postnatal, pubertal and adult testicular development. Food Chem. Toxicol. 42, 29-36.

Cappelletti, V., Saturno, G., Miodini, P., Körner, W., Daidone, MG., 2003. Selective modulation of ER-beta by estradiol and xenoestrogens in human breast cancer cell lines. Cell Mol. Life Sci., 60, 567-576.

Dallegrave, E., Mantese, F.D., Oliveira, R.T., Andrade, A.J., Dalsenter, P.R., Langeloh, A., 2007. Pre- and postnatal toxicity of the commercial glyphosate formulation in Wistar rats. Arch. Toxicol. 81, 665-673.

Daruich, J., Zirulnik, F., Sof a Gimenez, M., 2001. Effect of the herbicide glyphosate on enzymatic activity in pregnant rats and their fetuses. Environ. Res. 85, 226-231.

Gasnier, C., Dumont, C., Benachour, N., Clair, E., Chagnon, M.C., Seralini, G.E., 2009. Glyphosate-based herbicides are toxic and endocrine disruptors in human cell lines. Toxicology 262, 184-191.

Hokanson, R., Fudge, R., Chowdhary, R., Busbee, D., 2007. Alteration of estrogen-regulated gene expression in human cells induced by the agricultural and horticultural herbicide glyphosate. Hum. Exp. Toxicol. 26, 747-752.

Jauhiainena, A., Räsänena, K., Sarantilaa, R., Nuutinena, J., Kangasa, J. 1991. Occupational exposure of forest workers to glyphosate during brush saw spraying work. Am. Ind. Hyg. Assoc. J. 52, 61-64.

Lavy, T., Cowell, J., Steinmetz, J., Massey, J., 1992. Conifer seedling nursery worker exposure to glyphosate. Arch. Environ. Contam. Toxicol. 22, 6-13.

Lin, N., Garry, V., 2000. In vitro studies of cellular and molecular developmental toxicity of adjuvants, herbicides, and fungicides commonly used in Red River Valley, Minnesota. J Toxicol Environ Health [Part A] 60, 423-439.

Mandel, J.S., Alexander, B.H., Baker, B.A., Acquavella, J.F., Chapman, P., Honeycutt, R., 2005. Biomonitoring for farm families in the farm family exposure study. Scan. J. Work Environ. Health 31, 98-104.

Marc, J., Mulner-Lorillon, O., Bell , R., 2004. Glyphosate-based pesticides affect cell cycle regulation. Biol. Cell 96, 245-249.

McDuffie, H., Pahwa, P., McLaughlin, J., Spinelli, J., Fincham, S., Dosman, J., Robson, D., Skinnider, L., Choi, N., 2001. Non-Hodgkin's lymphoma and specific pesticide exposures in men. Cancer Epidem. Biomar. 10, 1155-1163.

Miodini, P., Fioravanti, L., Di Fronzo, G., Cappelletti, V., 1999. The two phyto-oestrogens genistein and quercetin exert different effects on oestrogen receptor function. Br. J. Cancer 80, 1150.

Morton, M., Arisaka, O., Miyake, A., Evans, B., 1999. Analysis of phyto-oestrogens by gas chromatography-mass spectrometry. Environ Toxicol. Phar. 7, 221-225.

Murphy, P.A., Song, T., Buseman, G., Barua, K., Beecher, G.R., Trainer, D., Holden, J., 1999. Isoflavones in retail and institutional soy foods. J. Agric. Food Chem. 47, 2697-2704.

Nakajima, N., Nozaki, N., Ishihara, K., Ishikawa, A., Tsuji, H., 2005. Analysis of isoflavone content in tempeh, a fermented soybean, and preparation of a new isoflavone-enriched tempeh. J. Bioscience Bioeng. 100, 685-687.

Pink, J.J., Jordan, V.C., 1996. Models of estrogen receptor regulation by estrogens and antiestrogens in breast cancer cell lines. Cancer Res. 56, 2321.

Rajah, T.T., Du, N., Drews, N., Cohn, R., 2009. Genistein in the presence of 17β-estradiol inhibits proliferation of ERβ breast cancer cells. Pharmacology 84, 68-73.

Richard, S., Moslemi, S., Sipahutar, H., Benachour, N., Seralini, G., 2005. Differential effects of glyphosate and Roundup on human placental cells and aromatase. Environ. Health Perspect. 113, 716-720.

Safe, S., Papineni, S., 2006. The role of xenoestrogenic compounds in the development of breast cancer. Trends Pharmacol. Sci. 27, 447-454.

Seo, H.S., David G. DeNardo, Yves Jacquot, Ioanna Laïos, Doris Salazar Vidal, Carmen Rojas Zambrana, Guy Leclercq, Powel H. Brown, 2006. Stimulatory effect of genistein and apigenin on the growth of breast cancer cells correlates with their ability to activate ER alpha. Breast Cancer Res. Tr. 99, 121-134.

Setchell, KD and Cassidy, A. 1999. Dietary isoflavones: biological effects and relevance to human health. J Nutr. 129, 758S-767S.

Sharma, O.P., 2009. Development of Simple and Sensitive HPLC Method for Determination of Glyphosate Residues in Soybean. A thesis in Master of Science in Environmental Toxicology, Environmental Toxicology Program, Chulabhorn Graduate Institute,Thailand.

Singleton, D.W., Khan, S.A., 2003. Xenoestrogen exposure and mechanisms of endocrine disruption. Front. Biosci. 8, 110-118.

Solomon, K., Anad n, A., Carrasquilla, G., Cerdeira, A., Marshall, E., Sanin, L., 2007. Coca and poppy eradication in Colombia: Environmental and human health assessment of aerially applied glyphosate. Rev. Environ. Contam. Toxicol. 190, 43-125.

Sotoca, A.M., Van Den Berg, H., Vervoort, J., Van Der Saag, P., Str m, A., Gustafsson, J., Rietjens, I., Murk, A.J., 2008. Influence of cellular ER /ER ratio on the ER -agonist induced proliferation of human T47D breast cancer cells. Toxicol. Sci. 105, 303-311.

Speirs, V., Walker, R.A., 2007. New perspectives into the biological and clinical relevance of oestrogen receptors in the human breast. J. Pathol. 211, 499-506.

Walsh, L., McCormick, C., Martin, C., Stocco, D., 2000. Roundup inhibits steroidogenesis by disrupting steroidogenic acute regulatory (StAR) protein expression. Environ. Health Perspect. 108, 769-776.

Wang T.T., Sathyamoorthy, N. and Phang J. M., 1996. Molecular effects of genistein on estrogen receptor mediated pathways. Carcinogenesis.17, 271-275.

WHO, 1994. Glyphosate. Environmental Health Criteria No. 159. World Health Organization, Geneva. http://www.inchem.org/documents/ehc/ehc/ehc159.htm

Williams, A.L., Watson, R.E., DeSesso, J.M., 2012. Developmental and reproductive outcomes in humans and animals after glyphosate exposure: A critical analysis. J. Toxicol. Environ. Health [Part B] 15, 39-96.

Wilson, V., Bobseine, K., Gray Jr, L., 2004. Development and characterization of a cell line that stably expresses an estrogen-responsive luciferase reporter for the detection of estrogen receptor agonist and antagonists. Toxicol. Sci. 81, 69-77.

Figure captions

Figure 1. Concentration-effect relationship of E2 and glyphosate on human breast cancer T47D (A&B) and MDA-MB231cells (C&D). Cells were treated with varying concentrations ranging from 10^{-12} to 10^{-6} M of E2 and glyphosate. Cells viability were compared between in completed medium (A and C) and in hormone withdrawal medium as shown in B and D by using MTT assay at 24 h (n=3, * = $p \leq 0.05$, significantly different as compared to control).

Figure 2. Proliferative effects of E2 and glyphosate on human breast cancer T47D cells. T47D cells were treated with varying concentrations of E2 and glyphosate ranging from 10^{-12} to 10^{-6} M and co-incubation with ICI 182780 (1 or 10 nM). Cells were cultured in hormone withdrawal medium for 5 days prior treatments. The cell viability was detected by MTT assay at 24 h. Each point was plotted from the mean value of three independent experiments ± SE as shown in the graph. (* $p \leq 0.05$ significantly different as compared to glyphosate alone).

Figure 3 The effects of 17β-estradiol (E2), glyphosate, and glyphosate coincubation with ICI 182780 on ERE transcription activity in T47D-KBluc cells (A). Cells were cultured in E2 withdrawal medium for 5 days before the treatment in each experiment. ICI 182780 at the concentrations 1 and 10 nM were used. The experiment was observed at 24 hours treatment (n=3, * = $p \leq 0.05$, significantly different as compared to glyphosate alone). Glyphosate at 1nM suppressed to the E2 effects along varying concentrations (B) (n=4, * $p \leq 0.05$ significantly different as compared to glyphosate alone).

Figure 4. **The effects of glyphosate on ERα and β expression.** E2-withdrawal T47D cells were used. (A) 6 hour and (B) 24 hour incubation time showed specific band of ERα, ERβ (66 kDa) and β-actin (44 kDa), a representative sample from one experiment. Optical densities of specific band ERα and ERβ were determined from western blot and each band was normalized to the β-actin band. The normalized mean of three replications ± SE optical density values are shown in the histogram of ERα (C) and ERβ (D) with * = $p \leq 0.05$, ** = $p \leq 0.01$ significantly different as compared to control .

Figure 5. Cytotoxicity of genistein on cell growth and ERE activation in T47D cells were investigated in E2-withdrawal media within 24 h treatment time. Genistein exhibited the stimulation of cell growth in the range of 10^{-8} – 10^{-4} M. by MTT assay (A) and the ERE activation were increased in the range of 10^{-11} – 10^{-6} M (B). (n=3, * $p \leq 0.05$, significantly different as compared to control).

Figure 6. ERE-gene transcription activity of T47D-KBluc cells (A) in coincubation of glyphosate (Gly) and genistein (Gen). The effects were compared between varying concentration of genistein alone and in combination with glyphosate (Gly 10^{-11} + Gen 10^{-7} M, Gly 10^{-10} + Gen 10^{-6} M and Gly 10^{-9} + Gen 10^{-5} M). Three replications and SE were showed in graph.(* $p \leq 0.05$, significantly different as compared to compound alone). The effect of glyphosate and genistein on cell growth (B). Cell number of T47D cells were counted after 3 days incubation with compound alone and coincubation treatments (n=3, $p \leq 0.05$, * significantly different as compared to compound alone, # significantly different as compared to control).

Figure1

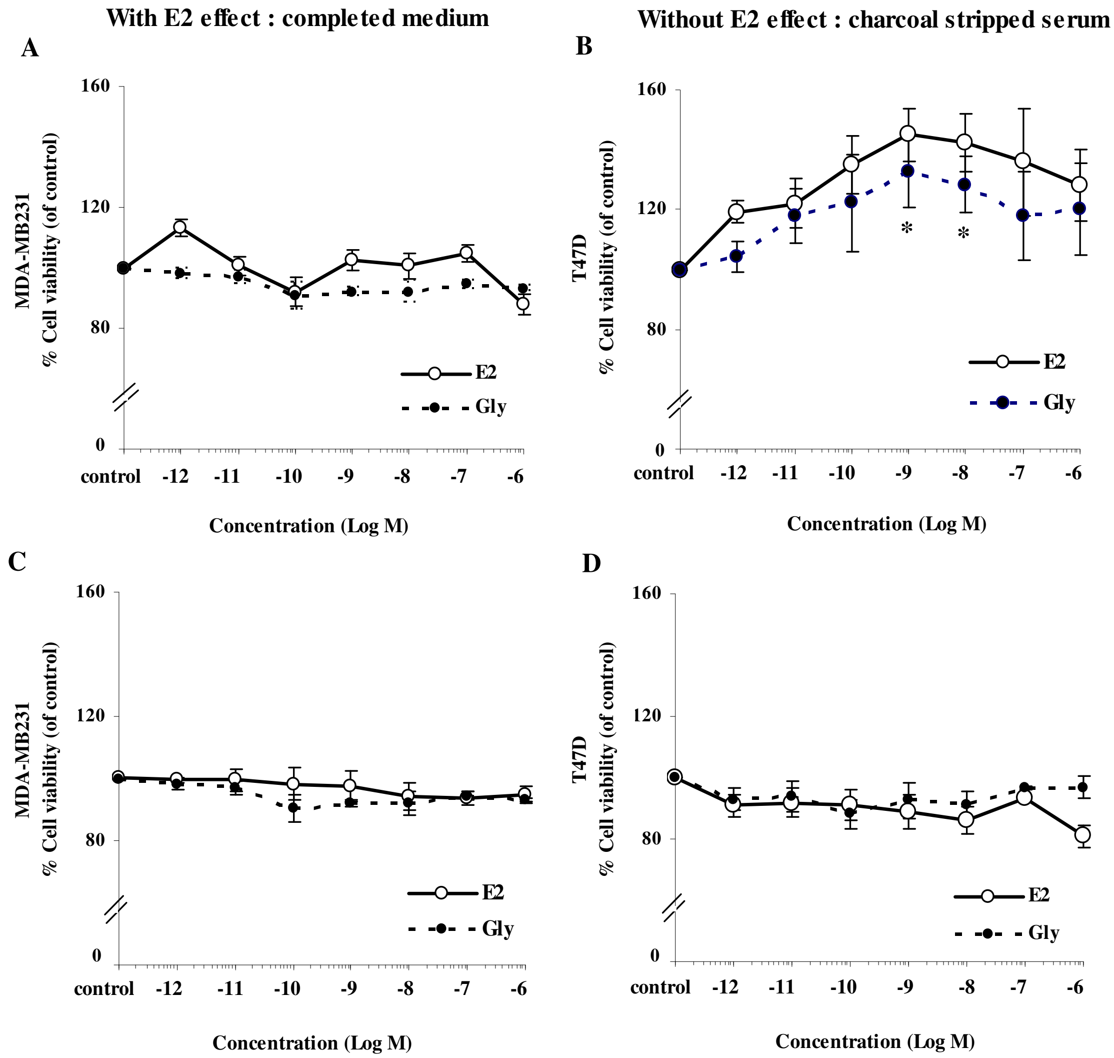

With E2 effect : completed medium
Without E2 effect : charcoal stripped serum
A
B
C
D
MDA-MB231
T47D
% Cell viability (of control)
160
120
80
0
control
-12
-11
-10
-9
-8
-7
-6
Concentration (Log M)
E2
Gly
*

Figure2

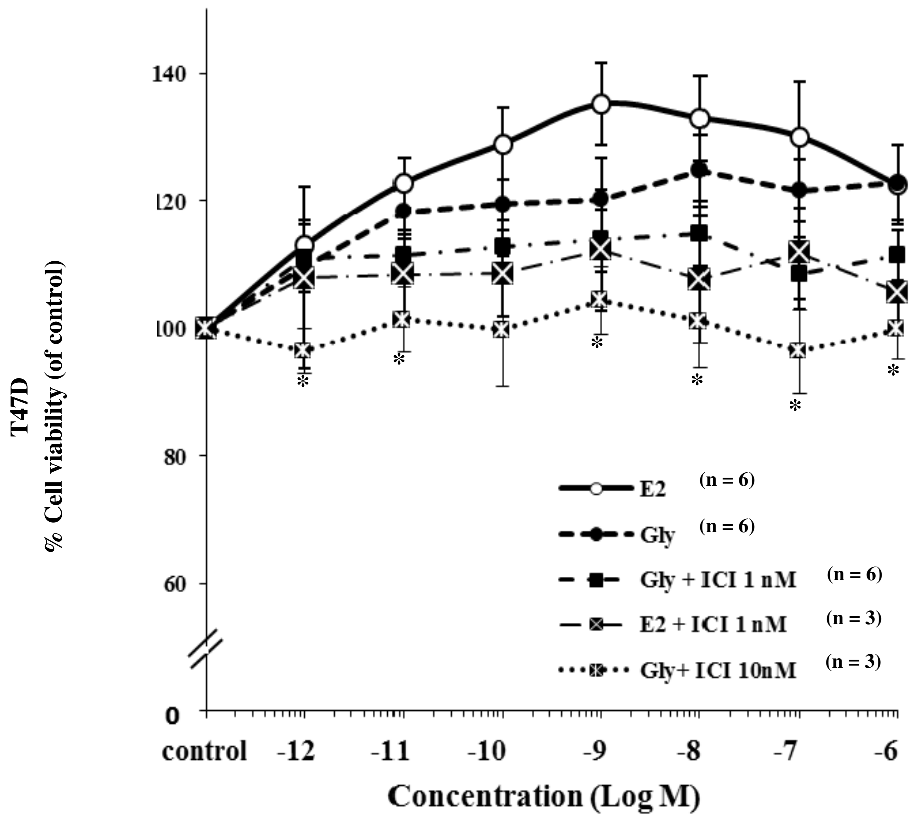
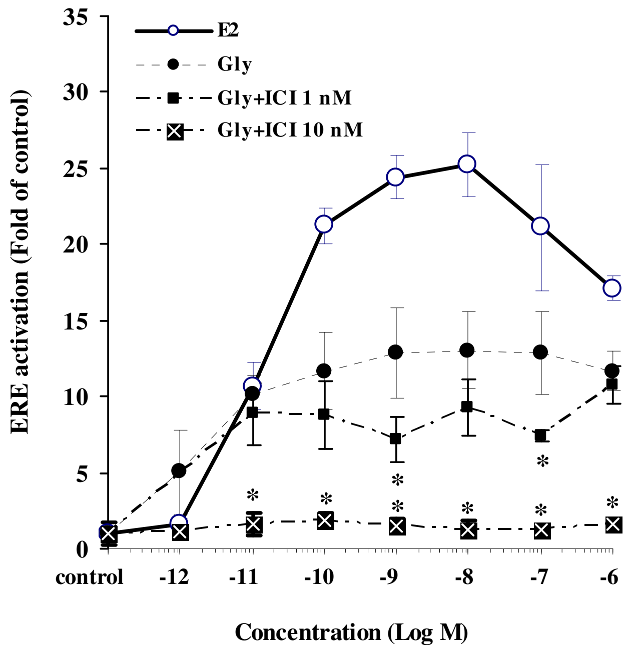

T47D
% Cell viability (of control)
140
120
100
80
60
0
control
-12
-11
-10
-9
-8
-7
-6
Concentration (Log M)
E2 (n = 6)
Gly (n = 6)
Gly + ICI 1 nM (n = 6)
E2 + ICI 1 nM (n = 3)
Gly+ ICI 10nM (n = 3)

Figure3

A


E2
Gly
Gly+ICI 1 nM
Gly+ICI 10 nM
ERE activation (Fold of control)
35
30
25
20
15
10
5
0
*
control
-12
-11
-10
-9
-8
-7
-6
Concentration (Log M)

B

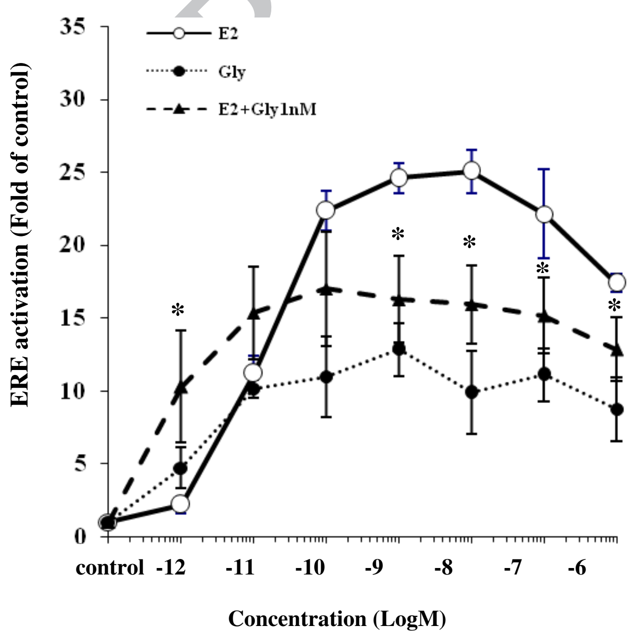

E2
Gly
E2+Gly1nM
ERE activation (Fold of control)
35
30
25
20
15
10
5
0
*
control
-12
-11
-10
-9
-8
-7
-6
Concentration (LogM)

Figure4

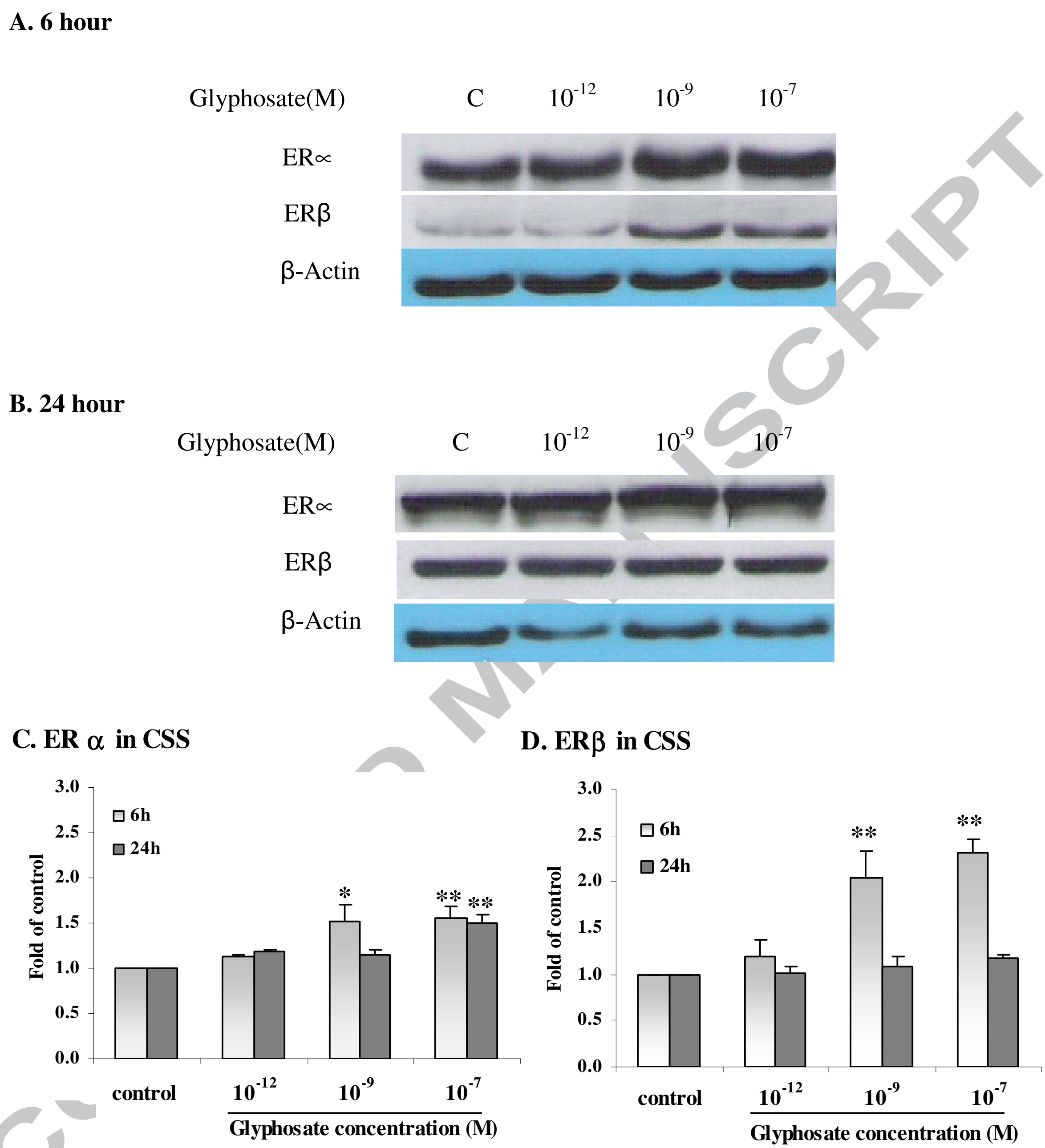
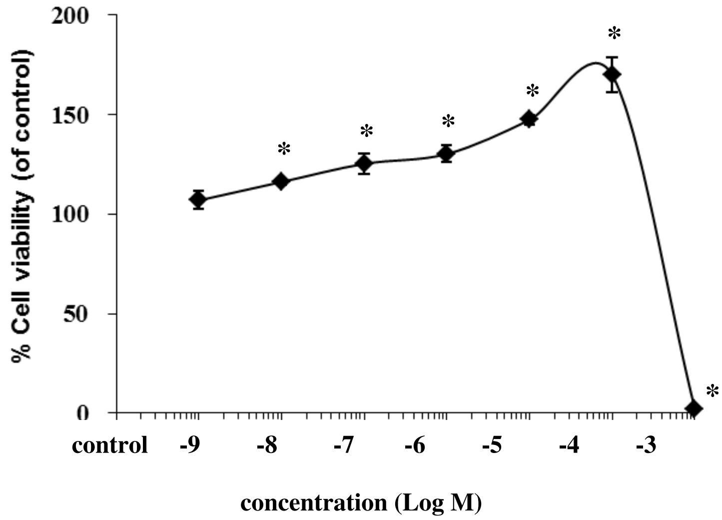

A. 6 hour
Glyphosate(M)
C
10-12
10-9
10-7
ER∝
ERβ
β-Actin
B. 24 hour
Glyphosate(M)
C
10-12
10-9
10-7
ER∝
ERβ
β-Actin
C. ER α in CSS
6h
24h
Fold of control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
** **
control
10-12
10-9
10-7
Glyphosate concentration (M)
D. ERβ in CSS
6h
24h
Fold of control
3.0
2.5
2.0
1.5
1.0
0.5
0.0
** **
control
10-12
10-9
10-7
Glyphosate concentration (M)

Figure5

A


200
150
100
50
0
% Cell viability (of control)
*
control
-9
-8
-7
-6
-5
-4
-3
concentration (Log M)

B

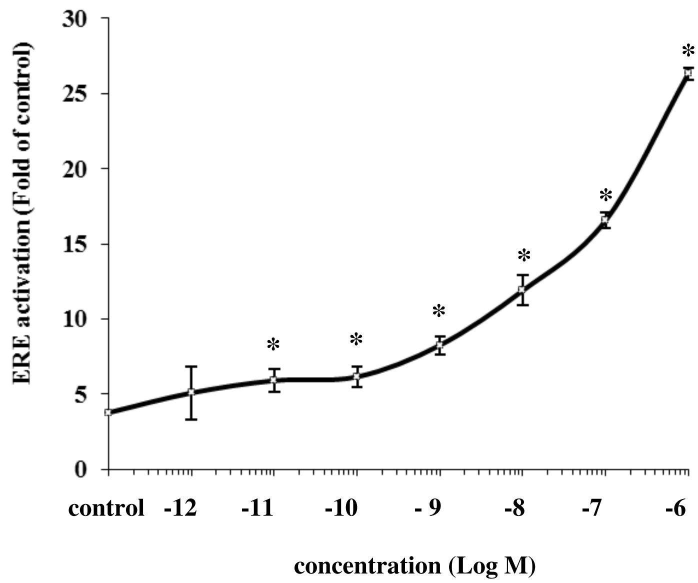
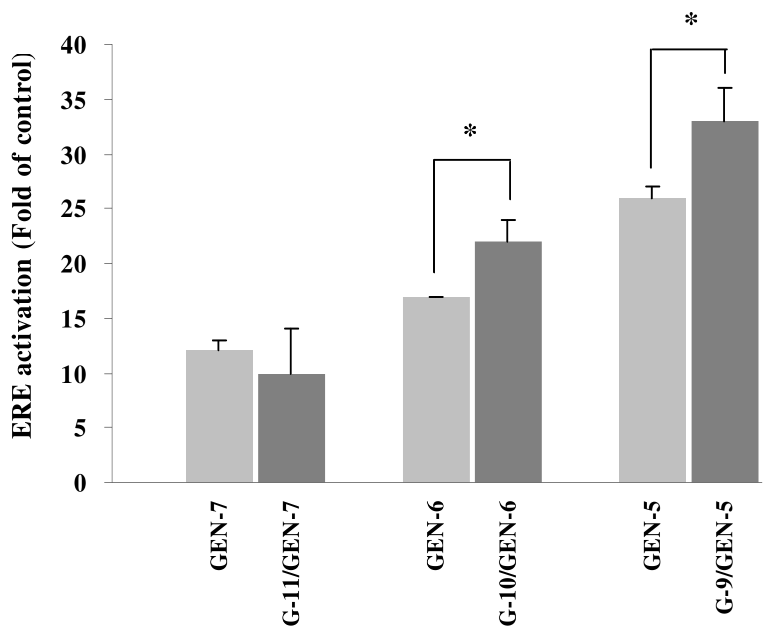
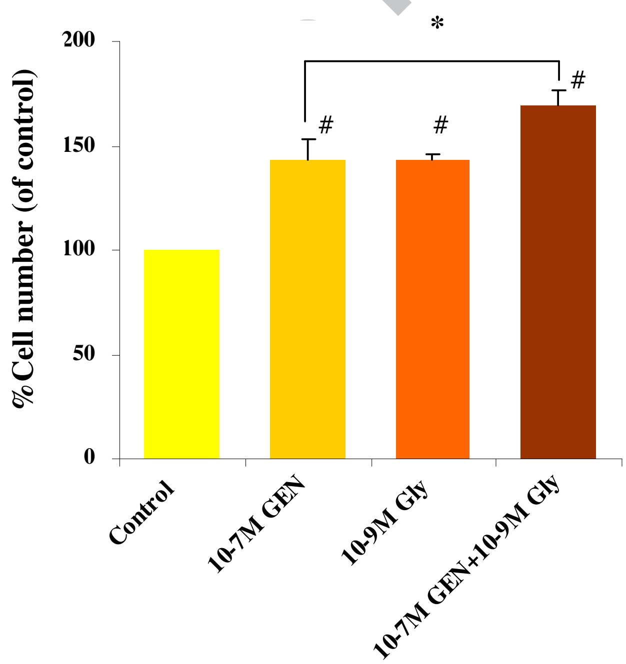

30
25
20
15
10
5
0
ERE activation (Fold of control)
*
control
-12
-11
-10
- 9
-8
-7
-6
concentration (Log M)

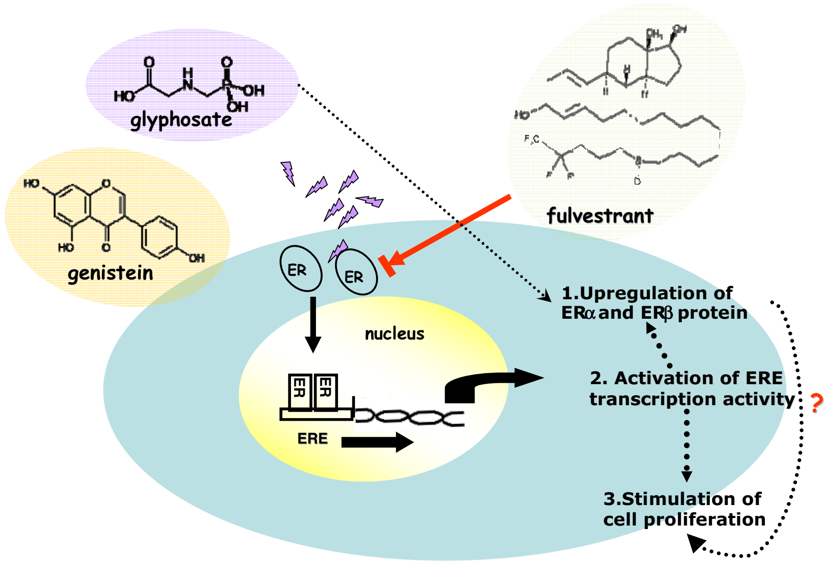
Figure6

A


ERE activation (Fold of control)
0
5
10
15
20
25
30
35
40
*
*
GEN-7
G-11/GEN-7
GEN-6
G-10/GEN-6
GEN-5
G-9/GEN-5

B


% Cell number (of control)
0
50
100
150
200
*
#
#
#
Control
10-7M GEN
10-9M Gly
10-7M GEN+10-9M Gly



Graphical abstract

Abtract

Glyphosate is an active ingredient of the most widely used herbicide and it is believed to be less toxic than other pesticides. However, several recent studies showed its potential adverse health effects to humans as it may be an endocrine disruptor. This study focuses on the effects of pure glyphosate on estrogen receptors (ERs) mediated transcriptional activity and their expressions. Glyphosate exerted proliferative effects only in human hormone-dependent breast cancer, T47D cells, but not in hormone-independent breast cancer, MDA-MB231 cells, at 10^{-12} to 10^{-6} M in estrogen withdrawal condition. The proliferative concentrations of glyphosate that induced the activation of estrogen response element (ERE) transcription activity were 5-13 fold of control in T47D-KBluc cells and this activation was inhibited by an estrogen antagonist, ICI 182780, indicating that the estrogenic activity of glyphosate was mediated via ERs. Furthermore, glyphosate also altered both ERα and β expression. These results indicated that low and environmentally relevant concentrations of glyphosate possessed estrogenic activity. Glyphosate-based herbicides are widely used for soybean cultivation, and our results also found that there was an additive estrogenic effect between glyphosate and genistein, a phytoestrogen in soybeans. However, these additive effects of glyphosate contamination in soybeans need further animal study.

Key words: glyphosate, estrogenic effect, genistein, human breast cancer, T47D, T47D-KBluc

Highlights

- Glyphosate at 10^{-12} to 10^{-6} M promoted growth of T47D cells via estrogen receptors.
- Glyphosate produced the activation of ERE which can be blocked by ICI 182780.
- Glyphosate altered estrogen receptors by increasing expression ratio of ERα and ERβ.
- Glyphosate had an additive effect with genistein on ERE activation and cell growth.